

Removal of Estrogens at Full and Pilot Scale Livestock Manure

Treatment Systems

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Keywords: Endocrine disruptor, Estrogens, Livestock manure, Reactor

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Abstract

Three experiments were conducted to 1) develop appropriate methods for livestock manure estrogen analysis; 2) determine estrogen removal in different manure treatment systems; and 3) determine estrogen removal from dairy manure in pilot scale reactors.

In Experiment I, the recoveries of 17 β -estradiol (E2) and estriol (E3) were evaluated in double distilled water and dairy manure after a base extraction and analysis of estrogens by enzyme-linked immunoassay (ELISA) and yeast estrogen screen (YES) assay. The recoveries of E2 were 104% (ELISA) and 97% (YES) in double distilled water. 112% of E2 and 79% of E3 in flushed dairy manure and 118% of E2 in anaerobic digester effluent were recovered with ELISA. 67% and 140% of E2 in flushed manure and anaerobic digester effluent, respectively, were recovered with YES assay.

In Experiment II, samples were collected from a full-scale manure handling system incorporating separation and aeration (Separation/Aeration), an anaerobic digester receiving dairy manure (Anaerobic Digester), and four conventional dairy and swine manure storages. 70% of E2 (230 vs. 769 $\mu\text{g}/\text{cow}/\text{day}$) and 86% of E3 (78 vs. 552 $\mu\text{g}/\text{cow}/\text{day}$) mass were removed from manure in the Separation/Aeration system when the effluent was compared to the influent; the ratio of E2 to total estrogenicity (E2-eq) averaged 76%. In the Anaerobic Digester, 38% of E2 (592 vs. 954 $\mu\text{g}/\text{cow}/\text{day}$) and 30% of E3 (338 vs. 483 $\mu\text{g}/\text{cow}/\text{day}$) mass were removed; E2 contributed more to E2-eq in the influent than in the effluent (43 vs. 26%). There was no

significant difference for E2-eq (431 vs. 284 ng/g of total solids) and E2 (248 vs. 73 ng/g of total solids) concentrations between barn and pit in conventional dairy manure storages; E2 contributed more to E2-eq in barn manure than in pit manure (54 vs. 30%). Concentrations of E2 and E2-eq in swine manure were highly variable between farms, but appreciable removal of these compounds was apparent following treatment with primary or primary plus secondary lagoons.

In Experiment III, samples were collected from six pilot scale reactors: two aerated reactors (60% and 100% aeration; AER60 and AER100), a nitrifying/denitrifying reactor (NDN), an enhanced biological phosphorus removal reactor (EBPR), an anaerobic digester (AD), and a nitrifying reactor (NI) following AD. The influent had higher mass of E2 and E2-eq than the effluent with all reactors. Estrogen removal efficiencies were expressed in two ways: % and %/aerobic hour (or hour) of the influent mass. Higher ammonia nitrogen removing reactors had higher E2 and E2-eq removal in %, higher E2 removal in %/aerobic hour, and the same E2-eq removal in %/aerobic hour compared to those with lower ammonia nitrogen removal. Estrogen removal efficiencies (both in % and %/aerobic hour) were similar in nitrifying and denitrifying reactors. Reactors with aeration supported greater estrogen removal than those without. Reactors with influent anaerobic digestion pretreatment had the same E2 and E2-eq removal in % but higher E2 and E2-eq removal in %/aerobic hour compared to those without.

In conclusion, the aerobic treatment systems removed more estrogens than the anaerobic ones, which means aerobic conditions support more estrogen degradation than anaerobic conditions. The change of the ratios of E2 to E2-eq in different livestock manure treatment systems reflected different removal rates of E2 and other estrogenic compounds especially with

anaerobic treatment. The pilot scale reactors significantly removed E2 and E2-eq in dairy manure. Aeration appears to be the key factor influencing E2 and E2-eq removal.

Keywords: Estrogens, livestock manure, reactor

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Chapter 1: Hormones in Waste from Concentrated Animal Feeding Operations

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Chapter 2: Methods for Estrogen Quantification in Livestock Manure

ABSTRACT

A method was developed for dairy manure estrogen extraction, which involved a two-step (alkaline plus toluene) procedure. The extraction efficiency was evaluated by determining the recoveries of 17 β -estradiol (E2) and estriol (E3) spiked into double distilled water and two manure sample matrices (flushed dairy manure and dairy manure effluent from an anaerobic digester) with both enzyme-linked immunosorbent assay (ELISA) and yeast estrogen screen (YES) assay. The quantity of estrogen detected was plotted and regressed against the quantity of estrogen spiked. The slopes of regression lines were considered as the method recoveries. E2 recovery was 104% (ELISA) and 97% (YES) in double distilled water. ELISA produced recoveries of 112% and 79% for E2 and E3 in flushed dairy manure, respectively; and 118% for E2 in anaerobic digester effluent. For the extraction method used in this study, YES produced fair recoveries of 67% and 140% for E2 in flushed dairy manure and anaerobic digester effluent, respectively. The two-step extraction procedure is appropriate for analysis of E2 and E3 in dairy manure, but further refinement may be needed before analysis of some wastes with the YES assay.

Keywords: Estrogens, dairy manure, ELISA, YES assay, recovery

INTRODUCTION

The determination of estrogens in the environment is not easy because of interference of complex environmental matrices and extremely low concentrations of the target compounds (Díaz-Cruz et al., 2003). Laborious and time-consuming procedures are required to achieve the sensitivity and selectivity necessary for estrogen analysis at physiologically active concentrations (ng/L) (Colborn et al., 1993). Researchers have been using a variety of methods for estrogen determination in different matrices, but reasonable recoveries have rarely been reported especially in livestock manure samples.

Estrogens need to be extracted first from the sample matrices and then must be assayed with proper methods. A variety of methods have been used for the extraction of estrogens. Diethyl ether was used for swine and dairy waste estrogen extraction (Vos, 1996; Raman et al., 2004). Hoffmann et al. (1997) developed a two-step extraction method (alkaline followed by toluene) for dairy cow urine and feces. Desbrow et al. (1998) and Cathum and Sabik (2001) used dichloromethane (DCM) for water sample extraction and 50% acetone in hexane followed by DCM for mussel samples.

Radio-immunoassay (RIA; Hoffmann et al., 1997; Gentili et al., 2002) and enzyme-linked immunosorbent assay (ELISA; Vos, 1996; Nichols et al., 1997; Finlay-Moore et al., 2000; Raman et al., 2004) have been used for assay of estrogens in environment samples. Both RIA and ELISA can detect estrogens at pg/ml concentrations in various kinds of sample matrices (water, urine, and feces), but RIA involves radiation hazard; ELISA is comparatively easy to perform but inferior to RIA in accuracy (Flemming and Bent, 2003). In recent years, spectrometry methods such as gas chromatography-mass spectroscopy (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS) have gained popularity for the determination of

estrogens in environmental samples (López de Alda and Barcelo, 2001). Although the sensitivity is poorer compared to immunoassay methods, spectrometry methods have advantage of less variability and greater selectivity (Flemming and Bent, 2003). Based on the availability of agents and equipments, the ELISA method was used in this research.

To determine total estrogenicity instead of a single estrogen compound as with the ELISA method, a bioassay method called yeast estrogen screen (YES) assay (Routledge and Sumpter, 1996; Holbrook et al., 2002) was incorporated into this research and compared with the ELISA method. The objective of this study was to develop an appropriate method for estrogen extraction and quantification with ELISA and YES assay in various livestock manure.

MATERIALS AND METHODS

The recoveries of E2 and E3 were tested in double distilled water and two dairy manure sample matrices with both ELISA and YES assays.

ELISA recovery test

To test E2 recoveries in double distilled water, one ml of double distilled water was spiked with different quantities of E2 (1, 5, 10, and 15 ng) and was extracted and assayed together with 1 ml of double distilled water as control with ELISA method.

To test E2 recoveries in dairy manure samples, one ml of liquid manure of main flush (MF; flushed barn manure) and flush water (FW; aerated effluent) collected in May 2006 from Virginia Tech Dairy Center and manure samples of anaerobic digester effluent (ADE) from a large commercial dairy collected in December 2005 were spiked with 0.75, 1.5, 3 ng, 0.56, 1.13, 2.25 ng, and 4.55, 8.33, and 11.54 ng of E2, respectively. The spiked quantity was chosen to reflect 50%, 100%, and 200% (for MF and FW) and 50%, 90%, and 125% (for ADE) of

expected E2 quantity in 1 ml of control samples (previously determined with ELISA). The spiked percent varied somewhat based on the actual control concentrations.

The E3 recovery was determined only in MF with 0.39, 0.78, and 1.56 ng of E3 spiked in 1 ml of MF sample, which was approximately 50%, 100%, and 200% of expected E3 in MF.

YES recovery test

To test E2-eq recoveries in double distilled water, 0.75, 1.5, 3, and 6 ng of E2 were spiked into each of 1 ml of double distilled water, and extracted and assayed together with 1 ml of double distilled water as control with YES assay.

To test E2-eq recoveries in dairy manure samples, one ml of MF and ADE was spiked with 0.78, 1.55, 2.29 and 23.81, 45.45, and 65.22 ng of E2, respectively.

Estrogen extraction

To extract estrogens in control or spiked samples, one ml of double distilled water or homogenized (magnetic stir plate) liquid manure sample was vortexed with 1.5 ml of chloroform and 1.5 ml of NaOH (1 M) and centrifuged for 20 min at 2500 x g (Hoffmann et al., 1997). One ml of the supernatant alkaline phase was removed and neutralized with 180 µl of acetic acid (90%, v/v). The neutralized sample was further extracted with 3 ml of toluene twice. The combined 6 ml of toluene was then evaporated to dryness with a gentle nitrogen gas stream.

Most estrogens and related compounds have pKa (acid-base ionization constant) values between 10-11 (Hurwitz and Liu, 1977), and undergo ionization when manure samples are extracted with 1 M NaOH solution (pH = 14), and become soluble in the alkaline phase. When the alkaline phase is neutralized with acetic acid, the decrease of pH to about 4.4 makes estrogens soluble again in the organic phase. Finally toluene is used to extract all acidified estrogens.

Estrogen analysis

The concentrations of E2 and E3 in control or spiked samples were determined with commercial ELISA kits (Assay Designs, MI, USA). The kits use a polyclonal antibody to bind, in a competitive manner, free estrogens in the standards and samples, or enzyme labeled estrogens contained in the kits. The dried sample extract was resuspended with 300-1000 μL of an assay buffer solution contained in the kits and a 100 μL aliquot was added to the wells in duplicates together with 50 μL of enzyme-labeled estrogens and antibodies. After a simultaneous incubation (2 hours) at room temperature on a plate shaker (500 rpm) the excess reagents were washed away and substrates for the enzymes were added for color development. After another incubation (45 minutes) without shaking the color reaction was stopped and the color change was quantified by measuring absorbance at 405 nm wavelength (MRX Revelation 4.22, Dynex Technology InC, Chantilly, VA). Every plate contained six E2 (29-30,000 pg/ml) or seven E3 (122-500,000 pg/ml) standards in duplicated wells from which standard curves were developed.

The concentrations of total estrogenicity (E2 equivalent; E2-eq) in control or spiked samples were determined with yeast estrogen screen (YES) assay described by Routledge and Sumpter (1996) with minor modifications. This assay consists of an estrogen-inducible expression system in a recombinant yeast strain (*Saccharomyces cerevisiae*). The human estrogen receptor gene was integrated into the yeast genome, which also contains expression plasmids carrying estrogen-responsive sequences controlling expression of the reporter gene *lac-Z*. Upon binding of active ligands to the human estrogen receptor, *lac-Z* is expressed to produce the enzyme β -galactosidase, which is secreted into the medium. It then metabolizes the chromogenic substrate, chlorophenol red- β -D-galactopyranoside (CPRG; Roche Diagnostics, Indianapolis, IN), from yellow into a red product.

All operations were conducted in a laminar fume hood (Contamination Control Inc, Lansdale, PA). In the 96-well sterile flat-bottom microtiter plates (Cat. 353072, BD Biosciences, San Jose, CA), 100 μ l of sample extract resuspended with absolute ethanol (500-1000 μ l) was added to wells for serial dilution across rows and 10 μ l from each dilution was transferred to blank wells in triplicates. After being evaporated to dryness in air, 200 μ l of growth medium containing yeast cells (grown to absorbance of 1 at 600 nm) was added to each well (Holbrook et al., 2002). The plates were incubated at 32 °C for 3 days and then 2 more days at room temperature. The color density was quantified by measuring absorbance at 575 nm (the highest absorption) and 620 nm (background) (Spectracount Microplate Photometer BS-10,000, Packard, Meriden, CT). Every plate contained two rows of E2 standard (>98% purity, Sigma Chemical Company, St. Louis, MO), ranging from 7.8 to 1000 pg/ml from which a standard curve was developed.

Statistical analysis

The quantity difference of E2, E3 or E2-eq detected between control and spiked double distilled water or manure samples was denoted Δ E2, Δ E3 or Δ E2-eq where:

$$\Delta (\text{E2, E3 or E2-eq; ng}) = \text{estrogens detected in spiked samples (ng)} - \text{estrogens detected in control samples (ng)}$$

Δ E2, Δ E3, and Δ E2-eq were plotted and regressed against the amount of estrogens spiked.

The regression model was described as $Y_{ij} = a X_{ij} + b + \epsilon_{ij}$, with Y was the expected quantity of estrogens detected (Δ E2, Δ E3, and Δ E2-eq), X was the quantity of estrogens spiked, and ϵ was the error term. There were three or four spiked quantities for each recovery test ($i = 1, 2, 3, 4$) with two duplicated assays for each spiked quantity ($j = 1, 2$). The regression slope (a) was considered to be the estrogen recovery, and the intercept (b) theoretically was the amount of

estrogens detected in double distilled water or estrogen-free sample matrices. Data were expressed as Mean \pm SD (n=2).

RESULTS AND DISCUSSION

ELISA recovery

The E2 concentration in double distilled water was below the detection limit (29 pg/ml). The recovery of E2 spiked into double distilled water was 104% (Figure 2.1), while recoveries of E2 spiked into MF, FW, and ADE were 113% (Figure 2.2), 112% (Figure 2.3), and 118% (Figure 2.4), respectively. The inflated intercept (2.91 ng) associated with ADE, i.e. E2 detected with zero spiked amount, compared to those in double distilled water (0.35 ng) and manure samples of MF (0.04 ng) and FW (0.08 ng), may have resulted from the much more complex sample matrix of ADE. The total solids contents in MF, FW, and ADE, assayed according to the standard method of APHA (1998), were 1.6%, 1.1%, and 6.0%, respectively. Therefore, high solids may interfere with the estrogen extraction somehow together with other substances in manures. The E3 recovery in MF was 79% (Figure 2.5).

For environmental estrogen studies using ELISA quantification, the reported recoveries varied greatly depending on sample matrices, extraction methods, and ELISA kits used. Huang and Sedlak (2001) reported $79 \pm 26\%$ recovery of E2 in surface water and filtered wastewater effluent after solid-phase extraction (SPE) and purification with high-performance liquid chromatography (HPLC). Raman et al. (2004) determined E2 content in dairy and swine manure after extraction using an alkaline-ethyl ether procedure, and reported recoveries of about 120% and 170% in winter and summer samples, respectively. Hanselman et al. (2004) reported 92% recovery of E2 in flushed dairy manure samples extracted with ethyl ether, but in that study E2 was spiked into the reconstituted dried extracts rather than the original sample matrix. Based on

the authors' experience (data not shown), the alkaline extraction tends to produce higher E2 signal than direct extraction with organic solvents such as diethyl ether, toluene, and hexane.

Obviously it is necessary to confirm the ELISA result with spectrometry methods. Farré et al. (2007) compared ELISA and LC-MS/MS for E2 detection in well water, river water, and wastewater samples with and without SPE cleanup and enrichment, and obtained a fairly good agreement between the two methods, with a modest overestimation (63%) resulting from ELISA. Hirobe et al. (2006) also observed overestimation with ELISA (49%) for E2 in samples from sewage treatment plants with glass-fiber filtration and SPE compared with LC-MS/MS. In contrast, near perfect agreement between ELISA and GC-MS was reported by Raman et al. (2001), with only 4% overestimation of ELISA ($R^2 = 0.93$), when E2 was determined in dairy waste solids after extraction with ethyl ether. The extraction methods and sample types may have effects on recoveries of quantification methods.

YES recovery

The E2-eq concentration in double distilled water was below the detection limit (15-30 pg/ml). In water, 97% of spiked E2 was recovered as (Figure 2.6). The recoveries in MF and ADE were 67% (Figure 2.7) and 140% (Figure 2.8), respectively. The inflated recovery in ADE seemed to be reasonable because of its complicated matrix, but the 67% recovery in MF was not expected. The biological explanation for this low recovery is not apparent, but perhaps compounds in MF quenched the estrogenic response of E2.

The YES assay was first applied by Routledge and Sumpter (1996) to test the estrogenic activity of surfactants and their major degradation products. Since then, this method has been used for biological and environmental samples, but the recovery of E2 associated with extraction and quantification was rarely reported. Burdge et al. (1998) reported 90% recovery of spiked E2

in bovine plasma extracted with diethyl ether. Gibson et al. (2005) determined total estrogenicity in the bile of fish exposed to effluent from wastewater treatment, and the recovery was $81 \pm 7\%$ after SPE and HPLC fractionation. The recoveries in environmental samples (wastewater, soil, and manure) have rarely been reported (Colucci et al., 2001; Holbrook et al., 2002; Burnison et al., 2003).

CONCLUSIONS

The recoveries of E2, E3, and E2-*eq* with ELISA and YES assay were evaluated in double distilled water and dairy manure based on a base-toluene extraction method. Except for 67% and 140% recovery of E2-*eq* in MF and ADE, respectively, all E2 and E3 recoveries ranged from were fairly close to 100%. The two-step extraction procedure is appropriate for analysis of E2 and E3 in dairy manure, but more work may be needed to improve recoveries in more complex matrices such as ADE.

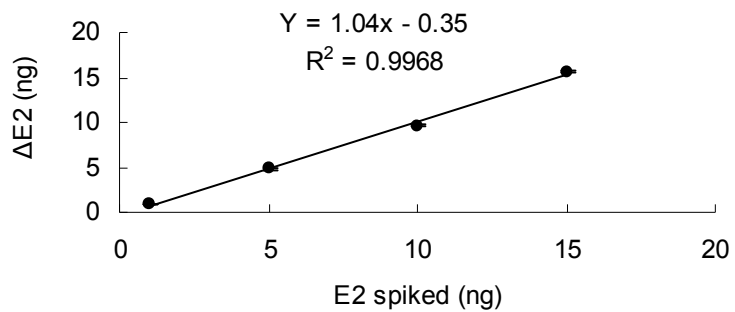


Figure 2.1 The E2 recovery in double distilled water with ELISA. Data are reported as Mean \pm SD (n=2). $\Delta E2 = E2$ detected in spiked samples - E2 detected in control samples.

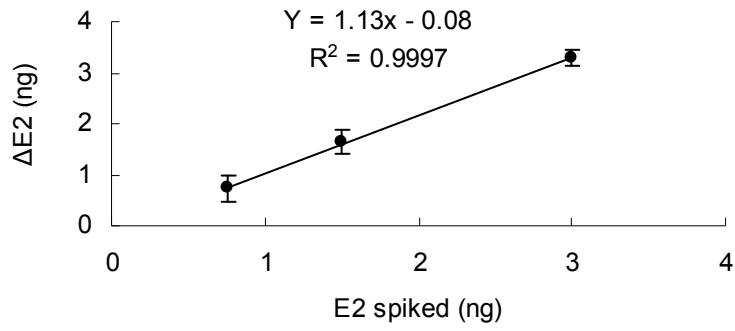


Figure 2.2 The E2 recovery in flushed dairy manure samples (main flush) with ELISA. Data are reported as Mean \pm SD (n=2). Δ E2 = E2 detected in spiked samples - E2 detected in control samples.

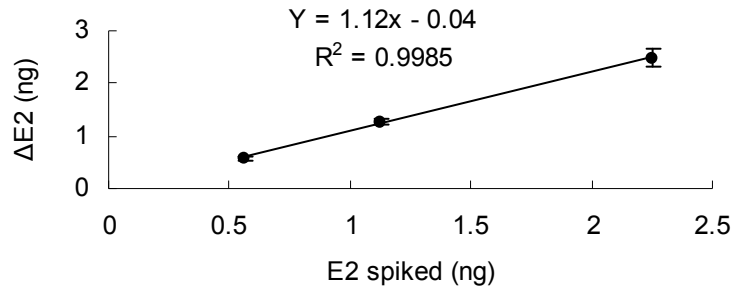


Figure 2.3 The E2 recovery in flushed dairy manure samples (flush water) with ELISA. Data are reported as Mean \pm SD (n=2). $\Delta E2 = E2$ detected in spiked samples - E2 detected in control samples.

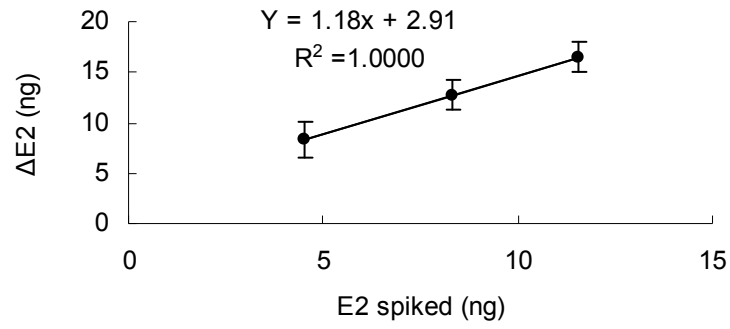


Figure 2.4 The E2 recovery in dairy manure samples of anaerobic digester effluent with ELISA.

Data are reported as Mean \pm SD (n=2). $\Delta E2 = E2$ detected in spiked samples - E2 detected in control samples.

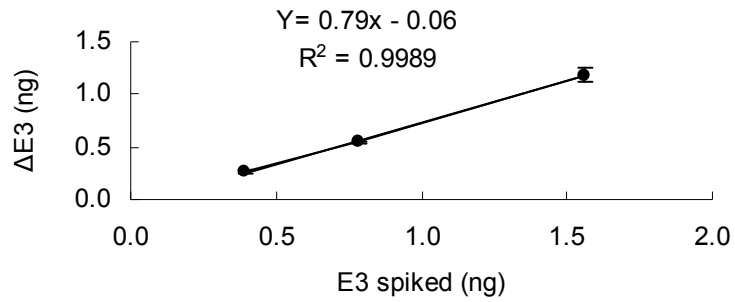


Figure 2.5 The E3 recovery in flushed dairy manure samples (main flush) with ELISA. Data are reported as Mean \pm SD (n=2). $\Delta E3 = E3$ detected in spiked samples – E3 detected in control samples.

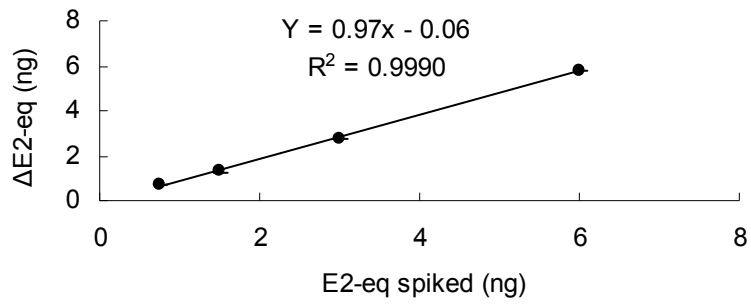


Figure 2.6 The E2-eq recovery in double distilled water with YES assay. Data are reported as Mean \pm SD (n=2). Δ E2-eq = E2-eq detected in spiked samples - E2-eq detected in control samples.

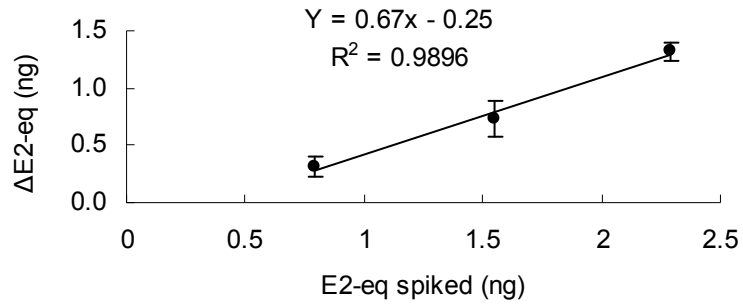


Figure 2.7 The E2-eq recovery in flushed dairy manure samples (main flush) with YES assay.

Data are reported as Mean \pm SD (n=2). Δ E2-eq = E2-eq detected in spiked samples - E2-eq detected in control samples.

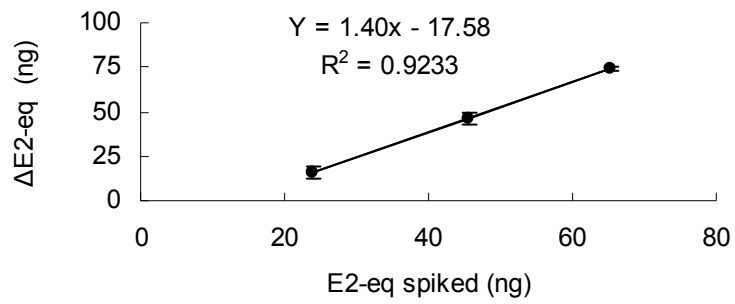


Figure 2.8 The E2-eq recovery in dairy manure samples of anaerobic digester effluent with YES assay. Data are reported as Mean \pm SD (n=2). Δ E2-eq = E2-eq detected in spiked samples - E2-eq detected in control samples.

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Chapter 3: Estrogen Removal in Livestock Manure Treatment Systems

ABSTRACT

Two experiments were conducted to determine the effects of different livestock manure treatment systems on removal of 17 β -estradiol (E2), estriol (E3), and total estrogenicity (E2 equivalent; E2-eq). In Experiment I, samples were collected monthly from a full-scale dairy manure handling system incorporating solid separation and liquid aeration (Separation/Aeration; 12 months) and a full-scale anaerobic digester receiving dairy manure (Anaerobic Digester; 9 months). In the Separation/Aeration system, the effluent had lower mass flow of E2 (230 vs. 769 $\mu\text{g}/\text{cow}/\text{day}$) and E3 (78 vs. 552 $\mu\text{g}/\text{cow}/\text{day}$) than the influent; 70% of E2 and 86% of E3 were removed. In Anaerobic Digester, the effluent had lower mass flow of E2 (592 vs. 954 $\mu\text{g}/\text{cow}/\text{day}$) and E3 (338 vs. 483 $\mu\text{g}/\text{cow}/\text{day}$) than the influent; 38% of E2 and 30% of E3 were removed.

In Experiment II, samples were collected monthly for three months from the Separation/Aeration system and the Anaerobic Digester, and from four conventional dairy (pit) and swine (lagoon) manure storages. In the Separation/Aeration system, the ratio of E2 to E2-eq averaged 76% across all sampling locations. In the Anaerobic Digester, E2 contributed more to E2-eq in the influent than in the effluent (43 vs. 26%). In conventional dairy manure storages, E2 contributed more to E2-eq in barn manure than in pit manure (54 vs. 30%). In sow farms, the E2 contribution to E2-eq decreased in lagoon manure (76 vs. 52%). In conclusion, E2 and E2-eq were substantially removed in different dairy and swine manure systems; the contribution of E2 to E2-eq did not change in the aerobic system but decreased in anaerobic manure storages.

Key words: Estrogen, livestock manure, treatment, removal

INTRODUCTION

In the last few decades, estrogens in the environment have raised great concern and interest for their potent endocrine disrupting effects. Significant estrogens have accumulated in the environment because of increased population and the intensity of animal production. At concentrations as low as ng per liter of water, estrogens can cause dangerous consequences to aquatic species (Colborn et al., 1993). For example, vitellogenesis and feminization in male fish are attributed to the presence of estrogenic compounds (Jobling et al., 1998).

To meet the strict federal and state laws regarding nutrient losses from farms, a variety of manure storage and treatment systems are implemented on livestock farms to decrease nutrient loading to the environment. These treatments include solid separation, liquid aeration, and anaerobic digestion, all of which may also affect the estrogen content of manure. Estrogen excretion by livestock in the United States was estimated at 49 tons annually (Lange et al., 2002), several-fold higher than was estimated for humans. Dairy cattle contribute the majority of these estrogens. It is important, therefore, to quantify the effects of manure treatments on estrogen content of livestock manure.

Most studies about estrogen removal have been done in municipal wastewater treatment plants (WWTPs). With conventional activated sludge systems (aeration) in municipal treatment plants, 17 β -estradiol (E2) was reduced from 15.6 ng/L in the influent to 1.8 ng/L in the effluent (Servos et al., 2005). When wastewater was incubated under anaerobic conditions with lake water and sediment, 99-176 μ g/L per day of E2 (5 mg/L originally) was transformed to estrone depending on the availability of electron acceptors (Czajka and Londry, 2006). Different degradation pathways (Rehm et al., 2001; Chakraborty and Coates, 2005; Seo et al., 2007; Heider, 2007), sorption of estrogens to biosolids (Andersen et al., 2005; Carballa et al., 2008),

release of free estrogens from conjugated forms (Shackleton et al., 1986; Jackson et al., 1992; D'Ascenzo et al., 2003), and/or possible formation of new estrogenic compounds (Holbrook et al., 2002) are the main factors influencing environmental estrogen removal.

Livestock wastes are very different in composition than municipal wastewater, with much higher solids content, and very different pollutants of concern, limiting the applicability of results from WWTPs. A few recent studies were conducted to determine estrogens in dairy and swine manure treatments. The E2 concentrations ranged from 1.8 to 49 and 0.8 to 27 ng/g (wet basis), respectively, in different types of swine and dairy manure treatment systems (Raman et al., 2004). Lorenzen et al. (2004) reported about 5 µg and 3 µg of total estrogenicity per g of dry weight in sow barn and lagoon manure. In swine manure wastewater, Furuichi et al. (2006) detected 1-1.5 ng/ml of E2, but total solids data were not available. Interpretation of literature data is difficult because most of the livestock manure estrogen studies have never reported recoveries of estrogen analysis and estrogen removal efficiencies.

The objective of this study was to determine estrogens and their removal efficiencies in different types of livestock manure treatment systems. Two experiments were conducted: I) to evaluate solid separation and liquid aeration and anaerobic digestion on concentrations, mass flow, and mass removal efficiencies of E2 and estriol (E3) in dairy manure; and II) to evaluate total estrogenicity and contribution of E2 to total estrogenicity in different dairy and swine manure treatment systems.

MATERIALS AND METHODS

Experiment I

Sample collection

Flushed dairy manure samples were collected from a full-scale manure handling system receiving manure from about 140 dairy cows at the Virginia Tech dairy center. This system uses a mechanical separator (concave screens with 0.79 mm screening opening) to separate manure liquids from solids, a short retention time anaerobic settling basin to further remove solids, and three aerated tanks in sequence. The total retention time in the system is approximately 180 days. The effluent from the third tank is reused to flush the barn, or is land-applied to crops via irrigation. This manure treatment system is denoted the Separation/Aeration system. The separator influent from the barn, separator effluent, settling basin effluent, and effluent from the three tanks were collected monthly from July 2005 to June 2006. The flush volume was measured indirectly via pressure transducers (Pressure Systems, Hampton, VA), which were installed on the bottom of each silo with data recorded on a CR-10 data logger (Campbell Scientific Inc., Logan, UT) for each silo. The liquid flow volume in other sampling locations was calculated by measuring flow tunnel area, flow time, flow height, and flow rate passing through tunnels to next stage of treatment. This allowed for calculation of estrogen mass flow.

The influent and effluent of a full-scale anaerobic digester on a large commercial dairy farm housing 2,400 cows were collected monthly from August 2005 to April 2006. The manure is continuously scraped and transferred to a double-walled anaerobic digester for 30 days at a constant temperature of 40 °C. The biogas produced from the digester is converted to electricity by generators and the heat from the generator is passed through a heat exchanger and transferred via a pipe to the fermentation tank to heat the manure. After 30 days in the anaerobic digester the manure is separated into solid and liquid fraction through a separator. The liquid is sprayed onto the agricultural land. The average manure excretion was estimated to be 65 L per cow per day

(ASAE, 2005). This allowed for estimation of the daily total manure and estrogen flow through the digester.

All samples were stored in capped glass jars at -20 °C before further processing.

Sample extraction

Samples were extracted using the method described by Zhao et al. (2008). Briefly, one ml of homogenized (magnetic stir plate) liquid manure sample was thoroughly mixed with 1.5 ml of chloroform and 1.5 ml of NaOH (1 M) in 12 * 75 mm glass tubes (Corning Life Sciences, Lowell, MA) and centrifuged for 20 minutes at 2500 x g. One ml of the supernatant alkaline phase was removed and neutralized with 180 µl of 90% acetic acid, and was further extracted with 3 ml of toluene twice. The combined 6 ml of toluene was then evaporated to dryness with a gentle nitrogen gas stream and stored at -20 °C for final assays.

Estrogen analysis

The concentrations of E2 and E3 in all samples were assayed with commercial enzyme-linked immunosorbent assay (ELISA) kits (Zhao et al., 2008). The kits use a polyclonal antibody to bind, in a competitive manner, free estrogens in the standards and samples, or enzyme labeled estrogens contained in the kits (Assay Designs, Ann Arbor, MI). The dried sample extract was resuspended with 300-1000 µL of an assay buffer solution contained in the kits and a 100 µL aliquot was added to the wells in duplicates together with 50 µL of enzyme-labeled estrogens and antibodies. After a simultaneous incubation (2 hours) at room temperature on a plate shaker (500 rpm) the excess reagents were washed away and the substrates for enzymes was added for color development. After another incubation (45 minutes) without shaking the color reaction was stopped and the color change was quantified by measuring absorbance at 405 nm wavelength with microtiter plate reader (MRX Revelation 4.22, Dynex Technology InC, Chantilly, VA).

Every plate contained six E2 standards (29-30,000 pg/ml) and seven E3 standards (122-500,000 pg/ml) in duplicated wells from which standard curves were developed.

Statistical analysis

The effects of the Separation/Aeration system on the concentrations and mass flow of E2 and E3 were analyzed according to the following model:

$$Y_{ijk} = \mu + S_i + L_j + SL_{ij} + e_{ijk},$$

where

μ = overall mean,

S_i = season effects ($i = 1, 2, 3, 4$);

L_j = location effects ($j = 1, 2, 3, 4, 5, 6$),

SL_{ij} = interaction between seasons and locations, and

e_{ijk} = residual error ($k = 1, 2, 3$).

In this system, samples were collected at six sampling locations monthly with data grouped to four seasons: summer (July, August, and September), fall (October, November, and December), winter (January, February, and March), and spring (April, May, and June).

The effects of the Anaerobic Digester on the concentrations and mass flow of E2 and E3 were analyzed according to the same model as the Separation/Aeration system, with two sampling locations ($i = 1, 2$) and three seasons ($j = 1, 2, 3$): fall (August, September, and October), winter (November, December, and January), and spring (February, March, and April).

All data were analyzed using the GLM procedure of SAS (SAS Institute, 2004). Data were reported as least square means (LSM) \pm standard error (SE). LSM were tested with Tukey pairwise comparisons and differences were declared significant at $P < 0.05$.

Experiment II

Sample collection

In the Separation/Aeration system described in Experiment I, the separator influent, separator effluent, settling basin effluent, and effluent from tank 3 were collected monthly in April, May and June of 2007. In the Anaerobic Digester described in Experiment I, the influent and effluent manure samples were collected monthly in April, May, and June of 2007.

To evaluate estrogen removal by conventional dairy manure treatment systems, fresh (scraped from the barn) and pit (concrete or earthen) manure samples were collected once during summer of 2007 from each of four commercial dairy farms housing 100-300 dairy cows. Three farms were located in Virginia and one in West Virginia. The scraped manure together with milking parlor wash water flowed to the pit and was stored there for 1-5 months before being emptied. Samples were collected when storage systems were emptied.

To evaluate estrogen removal by conventional swine manure treatment systems, barn (shallow pull-plug flushed with lagoon water) and anaerobic lagoon (primary and/or secondary) manure samples were collected once during summer of 2007 from two sow farms and two finishing farms in Virginia. All samples were stored in capped glass jars at -20 °C before further processing.

Sample extraction

As in Experiment I.

Estrogen analysis

The concentrations of E2 were analyzed as in Experiment I. The concentrations of total estrogenicity expressed as E2 equivalent (E2-eq) in all samples were determined using yeast estrogen screen (YES) assay as described by Zhao et al. (2008). This assay consists of an estrogen-inducible expression system in a recombinant yeast strain (*Saccharomyces cerevisiae*).

The human estrogen receptor gene was integrated into the yeast genome, which also contains expression plasmids carrying estrogen-responsive sequences controlling expression of the reporter gene *lac-Z*. Upon binding of active ligands to the human estrogen receptor, *lac-Z* is expressed to produce the enzyme β -galactosidase, which is secreted into the medium. It then metabolizes the chromogenic substrate, chlorophenol red- β -D-galactopyranoside (CPRG) (Roche Diagnostics, Indianapolis, IN), from yellow into a red product.

All operations were conducted in a laminar fume hood (Contamination Control Inc, Lansdale, PA). In the 96-well sterile flat-bottom microtiter plates (Cat. 353072, BD Biosciences, San Jose, CA), 100 μ l of sample extract resuspended with absolute ethanol (0.5-1.0 ml) was added to wells for serial dilution across rows and 10 μ l from each dilution was transferred to blank wells in triplicates. After being evaporated to dryness in air, 200 μ l of growth medium containing yeast cells (grown to absorbance of 1 at 600 nm) was added to each well (Holbrook et al., 2002). The plates were incubated at 32 °C for 3 days and then 2 more days at room temperature. The color density was quantified by measuring absorbance at 575 nm (the highest absorption) and 620 nm (background) (Spectracount Microplate Photometer BS-10,000, Packard, Meriden, CT). Every plate contained two rows of E2 standard (>98% purity, Sigma Chemical Company, St. Louis, MO) ranging from 7.8-1000 pg/ml from which a standard curve was developed.

All E2 and E2-eq data (ng/L) were normalized based on the total solids (TS) concentration (g/L) in the manure samples, and expressed as ng of E2 or E2-eq per g of TS (ng/g TS). The TS concentrations were determined according to the standard method (APHA, 1998).

Statistical analysis

In the Separation/Aeration system, the concentrations of E2-eq and E2, and ratios of E2 to E2-eq were analyzed according to the following model:

$$Y_{ij} = u + L_i + e_{ij},$$

where

u = overall mean,

L_i = sampling location effect ($i = 1, 2, 3, 4$),

e_{ij} = residual error ($j = 1, 2, 3$).

In this system, samples were collected at four sampling locations monthly for three months. For the Anaerobic Digester, data were analyzed using the same model with two sampling locations ($i = 1, 2$) and three monthly replications ($j = 1, 2, 3$). All data were analyzed using the GLM procedure of SAS (SAS Institute, 2004). Data were reported as $LSM \pm SE$; LSM were tested with Tukey pairwise comparisons and differences were declared significant at $P < 0.05$.

In conventional dairy manure storages, the concentrations of E2 and E2-eq were analyzed using the following model: $Y_{ij} = u + F_i + L_j + F*L_{ij}$, with u was the overall mean, F was the farm effect ($i = 1, 2, 3, 4$), L was the sampling location effect (barn and pit; $j = 1, 2$), and the interaction ($F*L$) was the error term. In conventional swine manure storages, E2-eq and E2 concentrations were reported as analytical mean ($n = 2$) without further statistical analysis because each farm was sampled just once.

RESULTS AND DISCUSSION

Experiment I

The Separation/Aeration system

Both seasons and sampling locations had significant effects on E2 concentrations (Figure 3.1 and Figure 3.3) and E2 mass flow per cow per day (Figure 3.2 and Figure 3.4) in the Separation/Aeration system. There was no significant effect of the interaction between seasons and sampling locations. The spring season had the highest E2 concentration (1045 pg/ml) and mass flow (852 $\mu\text{g}/\text{cow}/\text{day}$), and the lowest values were observed in the fall season (149 pg/ml and 128 $\mu\text{g}/\text{cow}/\text{day}$). There were no significant differences between the winter and summer season for E2 concentration (average 623 pg/ml) or mass flow (average 506 $\mu\text{g}/\text{cow}/\text{day}$). The duration of sample storage was confounded with seasonal effects, because the fall and winter samples were stored at -20°C for 1-6 months before processing and analysis; this is also true for the Anaerobic Digester samples described below.

Among the sampling locations in the Separation/Aeration system, there were no significant differences between separator influent, separator effluent, and settling basin effluent for E2 concentrations (average 763 pg/ml) or mass flow (average 655 $\mu\text{g}/\text{cow}/\text{day}$). The E2 concentrations (average 456 pg/ml) and mass flow (average 341 $\mu\text{g}/\text{cow}/\text{day}$) were lower in the three aerated tanks than in the original material, but the three tanks were not different from each other. Tank 3 effluent had significantly lower E2 concentration (321 vs. 890 pg/ml) and mass flow (230 vs. 769 $\mu\text{g}/\text{cow}/\text{day}$) than separator influent; 70% of E2 was removed through the whole system.

Seasons, sampling locations and the interaction between them all had significant effects on E3 concentrations and daily mass flow. The interaction was due to varied effects of tank 1 on E3 concentrations and mass flow across seasons. E3 concentration and mass flow were reduced in tank 1 to a greater extent in fall than in other seasons. The spring season had the highest E3 concentration and mass flow (825 pg/ml and 667 $\mu\text{g}/\text{cow}/\text{day}$; Figure 3.1 and Figure 3.2), and the

lowest values were observed in summer and fall (not different from each other). Among the sampling locations, separator influent and effluent had no significant difference for E3 concentration (average 561 pg/ml) or mass flow (average 479 $\mu\text{g}/\text{cow}/\text{day}$) (Figure 3.3 and Figure 3.4). The serial aeration significantly reduced both E3 concentration (121 vs. 395 pg/ml) and mass flow (78 vs. 337 $\mu\text{g}/\text{cow}/\text{day}$) when tank 3 and settling basin effluent were compared. 86% of E3 was removed throughout the system (separator influent vs. tank 3 effluent).

The estrogen removal efficiency in the Separation/Aeration system was comparable to the reported efficiencies by WWTPs, although the treatment processes and environmental conditions were different. Servos et al. (2005) reported 97% removal efficiencies of E2 in two municipal WWTPs incorporating primary and secondary treatment (high rate oxygen activated sludge). The removal efficiencies of E2, E1, and E3 were 99, 98, and 89%, respectively, by an activated sludge municipal WWTP (Chimchirian et al., 2007). 53-93% of E2 and 97-100% of E3 were removed by ten WWTPs with conventional activated sludge operation (Hashimoto et al., 2007).

The Anaerobic Digester

In the Anaerobic Digester, seasons had no significant effect on E2 concentrations and mass flow (Figure 3.5 and Figure 3.6). The influent had significantly higher E2 concentration (15.9 vs. 9.9 ng/ml) and mass flow (954 vs. 591 $\mu\text{g}/\text{cow}/\text{day}$) than the effluent (Figure 3.7 and Figure 3.8); 38% of E2 in the influent was removed through anaerobic digestion. There was no significant interaction between season and location.

Both season and sampling location had significant effects on E3 concentrations and mass flow. The interaction between them was not significant. The spring season had the highest E3 concentration (9.8 ng/ml) and mass flow (588 $\mu\text{g}/\text{cow}/\text{day}$), and there were no significant differences between winter and fall for both measurements (average 5.4 ng/ml and 323

µg/cow/day). The influent had significantly higher E3 concentration (8.0 vs. 5.6 ng/ml) and mass flow (483 vs. 338 µg/cow/day) than the effluent; 30% of E3 in the influent was removed through anaerobic digestion.

Literature data on estrogen removal during anaerobic digestion are rarely reported. Carballa et al. (2007) observed 88% removal of the sum of E2 and E1 from sewage sludge through a pilot scale conventional anaerobic digester; solid retention time (10-30 days) and temperature (37 or 55 °C) had no significant effects on estrogen removal. E2 was readily transformed to E1 and probably to 17 α -estradiol in anaerobic cultures established with lake water and sediments with different electron acceptors (CO₂, sulfate, iron, or nitrate); these conversions reduced total estrogenicity (Czajka and Londry, 2006). In contrast, E2 was formed from spiked E1 in anaerobic batching experiment with sewage sludge when nitrate was not available (Joss et al., 2004). Interestingly, an increase in the mass of total estrogenicity was reported in anaerobic digestion processes in two wastewater treatment systems (Holbrook et al., 2002); the authors concluded that this probably was due to released estrogenic compounds that were previously non-extractable.

The Separation/Aeration system (aerobic) had higher estrogen removal efficiencies than the Anaerobic Digester. Lorenzen et al. (2004) also reported more E2 degradation in municipal biosolids that had undergone aerobic digestion compared to anaerobic digestion. 88% of spiked E2 was degraded in 1 day in aerobic condition by an E2-degrading culture developed from activated sludge of sewage treatment plant; in contrast, only 50% of spiked E2 was degraded in 7 days in anaerobic condition (Lee and Liu, 2002). In batch experiments with sewage sludge, the degradation rate of E1 was increased by a factor of 3-5 in the transition from anaerobic to anoxic

as well as between anoxic and aerobic, and the E2 degradation rate also increased between anaerobic and aerobic by a factor of 3 (Joss et al., 2004).

Although the mechanistic explanation for faster estrogen degradation in aerobic conditions than in anaerobic conditions is unclear, the different degradation rates may result from different degradation pathways. Under aerobic conditions, the introduction of hydroxyl groups by mono- and dioxygenases, carboxylation, ring cleavage, and final decarboxylation are the basic steps for degradation of phenolic compounds (Rehm et al., 2001; Seo et al., 2007).

Anaerobes may degrade phenolic compounds through hydroxylation and carboxylation as the initial activation steps (Chakraborty and Coates, 2005; Heider, 2007). The aromatic rings need to be reduced to destabilize the resonant electron distribution. Next, the ring structure is broken through reductive cleavage to form aliphatic acids to produce metabolites suitable for methanogenic use (Evans, 1977; Grbic-Galic and Vogel, 1987).

More E2 was found than E3 in the influents of both the Separation/Aeration and Anaerobic Digestion systems, which is in agreement with other studies. Hanselmann et al. (2006) reported 643 pg/ml of E2 and below detection limit of E3 (< 250 pg/ml) in flushed dairy barn manure assayed with gas chromatography-mass spectroscopy (GC-MS). E3 was removed more efficiently than E2 by the aerobic treatment system (Separation/Aeration). Sarmah et al. (2000) detected 29-331 pg/ml of E2 and below detection limit of E3 (< 6 pg/ml, GC-MS) in dairy manure oxidation ponds; this obviously indicated higher removal of E3 than E2 in aerobic conditions, which is in agreement with the current study. There was no significant difference between E2 and E3 removal efficiencies by the anaerobic treatment system (Anaerobic Digester); this is in contrast to much faster degradation of E2 than E3 in an anaerobic digester that treated swine manure (Furuichi et al., 2006).

Experiment II

The Separation/Aeration system

Similar to the results from Experiment I, the sampling location had significant effects on concentrations of E2-eq and E2 (Figure 3.9). Separator influent was not different from separator effluent and settling basin effluent in concentrations of E2-eq (average 205 ng/g TS) and E2 (average 157 ng/g TS). The separator influent TS (2.9 vs. 1.4%) and E2 (157 vs. 108 ng/g TS) concentrations were higher than that collected during the same period of 2006 in Experiment I. Tank 3 effluent had significantly lower E2-eq (115 ng/g TS) and E2 (88 ng/g TS) concentrations than separator influent. Concentrations of E2-eq and E2 were decreased by 49% and 50%, respectively, across the system. Hanselmann et al. (2006) determined E1, E2, E3 (undetectable), and 17 α -estradiol (GC-MS) in flushed dairy barn manure after solid separation from a research farm based on samplings of five consecutive days. According to their data, the E2 concentration averaged 77 ng/g TS, ranging from 27 to 145 ng/g TS. The E2-eq concentration from the determined estrogens averaged 120 ng/g TS (42-190 ng/g TS), with 1.0, 0.38, and 0.04 of relative estrogenic potency being assigned to E2 and E1 (Rutishauser et al., 2004) and 17 α -estradiol (Hasenbrink et al., 2006), respectively. These values are very similar to those observed in the current study despite differences in treatment of flushed manure. The reported TS in that study was lower (0.9 vs. 2.9%; Hanselmann et al., 2006) than that of the separator influent (flushed barn manure) in the Separation/Aeration system.

The contribution of E2 to total estrogenicity was the same among the four sampling locations averaging 76% (Table 3.1). The similar contribution of E2 to total estrogenicity throughout the system implies that degradation and sorption of all estrogenic compounds in raw manure occurred at similar rates. In the study of Hanselmann et al. (2006) mentioned above, the

ratio of E2 to total estrogenicity calculated from E2, E1, and 17 α -estradiol averaged 66% (47-75%) in flushed dairy barn manure.

The Anaerobic Digester

The influent was not significantly different from the effluent in E2-eq concentration (average 1261 ng/g TS; Figure 3.10). E2 concentration was significantly lower in the effluent than in the influent (283 vs. 610 ng/g TS; 54% of decrease). The influent TS (9.8 vs. 6.0%) and E2 (610 vs. 383 ng/g TS) concentrations were higher than that collected during the same period of 2006 in Experiment I. Compared to separator influent of the Separation/Aeration system, both E2-eq and E2 concentrations were much higher in the anaerobic digester influent. The differences in animals, diets, manure characteristics, manure handling, and sampling techniques may contribute to the differences in estrogen concentrations. Lorenzen et al. (2004) reported as high as 3000 ng/g TS of E2-eq in lactating cow fresh manure based on ethyl acetate extraction. Even higher E2-eq concentrations (3300-14800 ng/g TS) were observed in scraped dairy manure (Ermawati et al., 2007), but the extraction method was not reported.

E2 contributed less to the total estrogenicity in the effluent than in the influent (26 vs. 43%; Table 3.1). This probably resulted from different degradation rates of E2 compared to other estrogenic compounds, hydrolysis of conjugated estrogens, or synthesis of new estrogenic compounds during digestion. The authors of this study also observed decreased ratios of E2 to total estrogenicity in dairy manure (influent vs. effluent) in a pilot scale anaerobic digester (37 vs. 22%; Chapter 4). Literature data on the effect of anaerobic digestion on the contribution of E2 to total estrogenicity in dairy manure are not available. According to a swine manure treatment study with up-flow anaerobic sludge blanket (UASB; essentially anaerobic digester with suspended sludge blanket), the contribution of E2 to total estrogenicity decreased from 27% in

the influent to 16% in the effluent (Furuichi et al., 2006). The authors calculated that E2 was removed the most (63%) by UASB compared to other natural estrogens E1 (30%), E3 (21%), and 17 α -estradiol (6%). This could explain the decreased ratio of E2 to total estrogenicity after anaerobic digestion in the current study, but the exact mechanism needs to be further clarified.

Conventional dairy manure storages

The E2-eq and E2 concentrations were not significantly different among farms (average 358 ng/g TS for E2-eq and 160 ng/g TS for E2) and sampling locations (barn vs. pit; 431 vs. 285 ng/g TS for E2-eq; 248 vs. 73 ng/g TS for E2). The reason that there was no difference between barn and pit was that great variation was observed among farms. Also all farms were sampled only once. The numerical decrease in E2-eq (34%) and E2 (71%) concentrations after pit storages was similar to the reduction observed in the Anaerobic Digester. Sampling technique and on-farm situations may influence manure estrogen concentrations more than manure storage system. Possibly in some manure samples bedding material and other particles increased the TS content without increasing estrogens at the same or greater extent. This is the biggest challenge in livestock manure sampling. Lorenzen et al. (2004) determined E2-eq (YES assay) content in fresh and pit manure from six dairy farms based on ethyl acetate extraction. The E2-eq concentrations ranged from 22 to 3000 ng/g TS in fresh and pit manure; interestingly, only one out of the six dairies had lower E2-eq concentrations in pit manure than in fresh barn manure. Higher manure E2-eq concentrations were also noticed in those farms with soy (containing phytoestrogens) feeding in cow diets than those without soy feeding (Lorenzen et al., 2004).

The E2 ratio to E2-eq was significantly higher in one conventional dairy manure storage (65%) than the other three (34%; not significantly different from each other). E2 contributed more to E2-eq in barn manure (average 54%) than in anaerobically stored pit manure (average

30%) in all farms (Table 3.1). As with the Anaerobic Digester, this may result from higher removal rate of E2 as compared to other estrogenic compounds (Furuichi et al., 2006). While our replication is limited, the similarity of estrogen transformation in the two types of anaerobic systems merits further research.

Conventional swine manure storages

The E2-eq and E2 concentrations in fresh barn manure and pit storages from the four commercial swine farms are listed in Table 3.3. Great variation was observed between farms. Sow farms had numerically higher E2-eq and E2 concentrations than finishing farms in barn manure. Raman et al. (2004) also reported higher E2 content in farrowing pits than in finishing lagoons. However, Lorenzen et al. (2004) observed lower E2-eq in manure of sow barn than that of finishing barn (4000 vs. 6000 ng/g TS).

Primary lagoons had numerically lower estrogen content than barn manure. Similarly, three times lower E2 content on a dry basis was found in sow lagoon than in pit (Raman et al., 2004). The secondary lagoons had numerically the lowest E2-eq and E2 concentrations in sow farm B, and were much lower than pit manure in finishing farm D (primary lagoon sampling not available). Extended anaerobic lagoon treatments consistently reduced estrogenicity in swine manure.

E2 accounted for 70% and 83% of E2-eq in fresh pit manure of sow farm A and B, respectively, and its proportion decreased in sow lagoon storages. This is similar as observed in conventional dairy manure storages. The E2 (mass spectrometry method) contribution to E2-eq (YES assay) in a flushed farrowing pit manure based on solid phase extraction was lower (47%) than those in the current study (Shappell et al., 2007); this may be explained by differences in estrogen quantification methods, sampling techniques, and manure systems. The contribution of

E2 to E2-eq decreased to 18% in primary lagoon in that study (Shappell et al., 2007). In the two finishing farms, E2 contributed to 33% and 34% of total estrogenicity in fresh pit manure, respectively. Furuichi et al. (2006) reported 23-30% of E2 (mass spectrometry method) in E2-eq (MVLN trans-activation assay) in finishing swine pit manure. These values were similar to the current study. The ratios of E2 to E2-eq increased in finishing lagoon manures (43% and 77%, respectively), but these values are questionable because of the low estrogen concentrations (close to detection limit) and TS content (as low as 1.3 g/L).

CONCLUSIONS

All dairy manure treatment and storage systems resulted in significant removal of E2, E3, or E2-eq. As has been commonly observed in WWTPs, the aerobic dairy manure treatment system had higher E2 and E3 removal efficiencies than the anaerobic one; this corresponds to more efficient estrogen degradation pathways in aerobic conditions than in anaerobic conditions. The concentrations of E2 and E2-eq decreased in conventional dairy and swine manure storages.

The removal rates of E2 and E2-eq in aerobic conditions were similar as indicated by the same ratios of E2 to E2-eq in aerobic manure treatments. In contrast, E2 was more efficiently removed than E2-eq in anaerobic treatments and storages, which is in agreement with other studies. This resulted from different estrogen degradation rates and/or possibly the release or formation of new estrogenic compounds in anaerobic conditions. Further research is needed to clarify estrogen degradation and removal mechanisms in different manure treatments and storages.

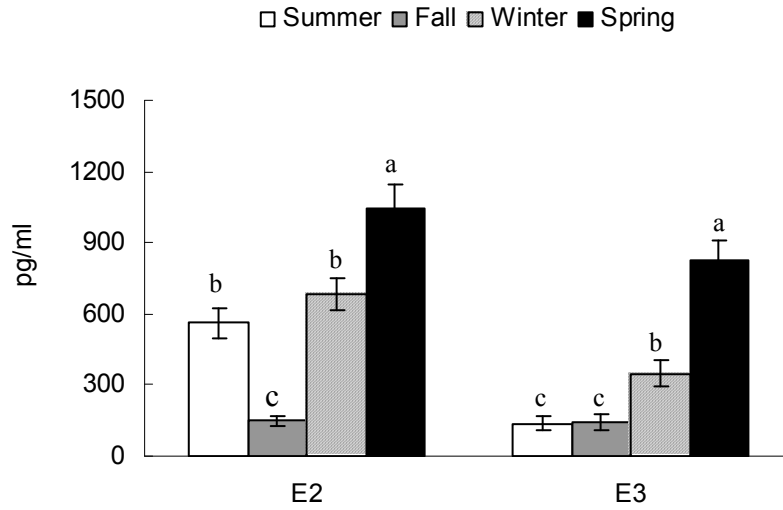


Figure 3.1 The seasonal effect on E2 and E3 concentrations (pg/ml) in the Separation/Aeration system. Data are reported as LSM \pm SE (n=18). The seasonal effect was significant. There was no effect of interaction of season and sampling location for E2. The interaction affected E3. Bars with different letters are significantly different ($P < 0.05$).

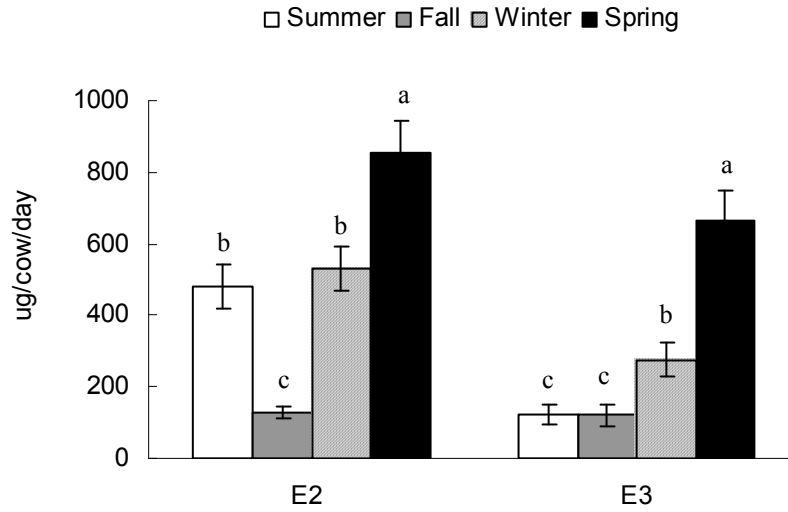


Figure 3.2 The seasonal effect on E2 and E3 mass flow ($\mu\text{g}/\text{cow}/\text{day}$) in the Separation/Aeration system. Data are reported as $\text{LSM} \pm \text{SE}$ ($n=18$). The seasonal effect was significant. There was no effect of interaction of season and sampling location for E2. The interaction affected E3. Bars with different letters are significantly different ($P < 0.05$).

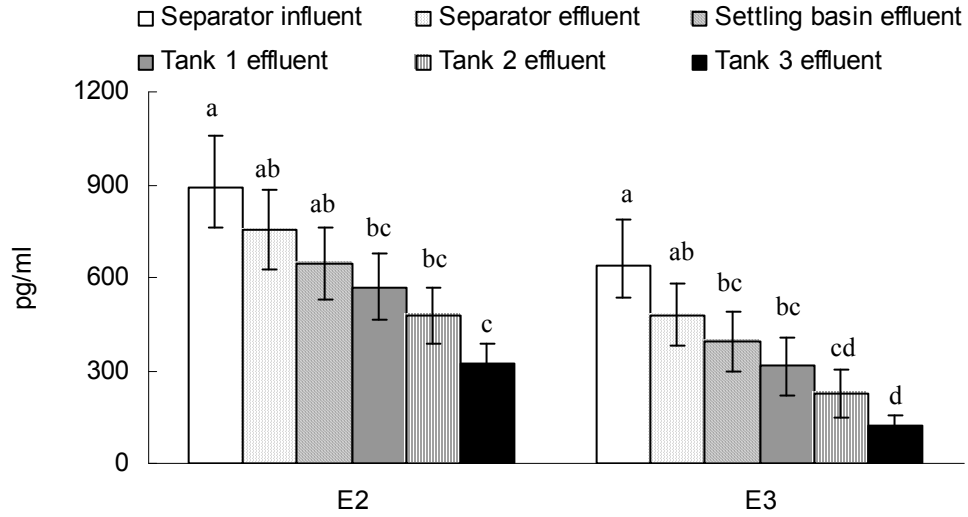


Figure 3.3 The location effect on E2 and E3 concentrations (pg/ml) in the Separation/Aeration system. Data are reported as LSM \pm SE (n=12). The location effect was significant. There was no effect of interaction of season and sampling location for E2. The interaction affected E3. Bars with different letters are significantly different ($P < 0.05$).

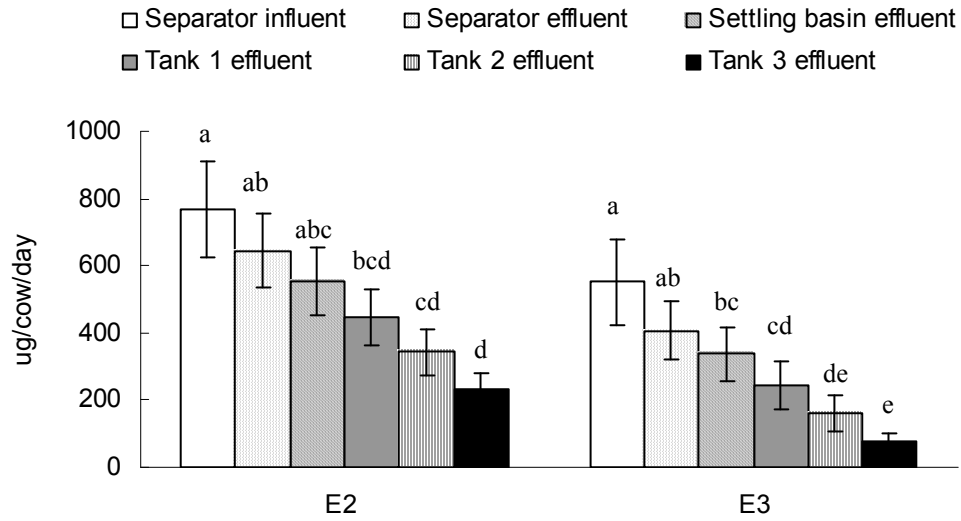


Figure 3.4 The location effect on E2 and E3 mass flow ($\mu\text{g}/\text{cow}/\text{d}$) in the Separation/Aeration system. Data are reported as $\text{LSM} \pm \text{SE}$ ($n=12$). The location effect was significant. There was no effect of interaction of season and sampling location for E2. The interaction affected E3. Bars with different letters are significantly different ($P < 0.05$).

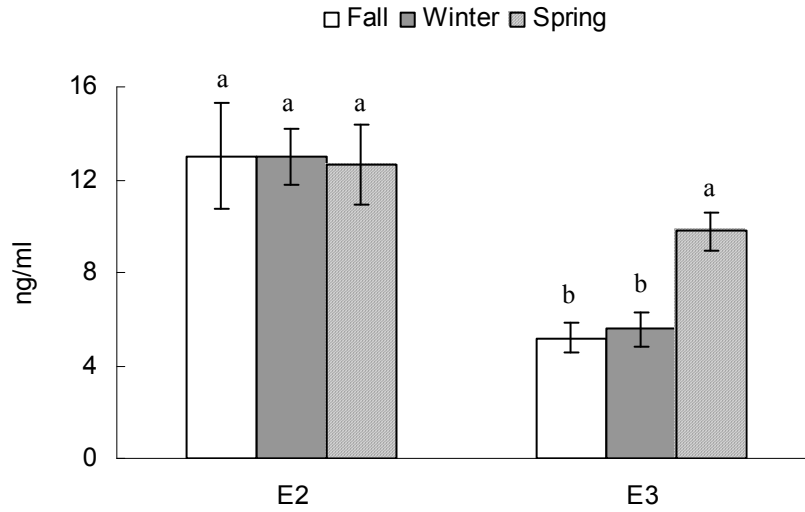


Figure 3.5 The seasonal effect on E2 and E3 concentrations (ng/ml) in the Anaerobic Digester. Data are reported as LSM \pm SE (n=6). The seasonal effect was not significant for E2. The season affected E3. There was no effect of interaction of season and sampling location. Bars with different letters are significantly different ($P < 0.05$).

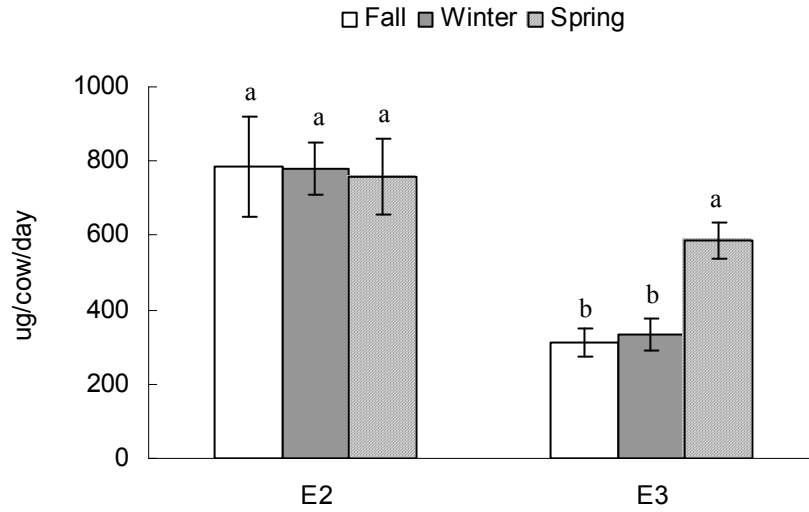


Figure 3.6 The seasonal effect on E2 and E3 mass flow ($\mu\text{g}/\text{cow}/\text{day}$) in the Anaerobic Digester. Data are reported as $\text{LSM} \pm \text{SE}$ ($n=6$). The seasonal effect was not significant for E2. The season affected E3. There was no effect of interaction of season and sampling location. Bars with different letters are significantly different ($P < 0.05$).

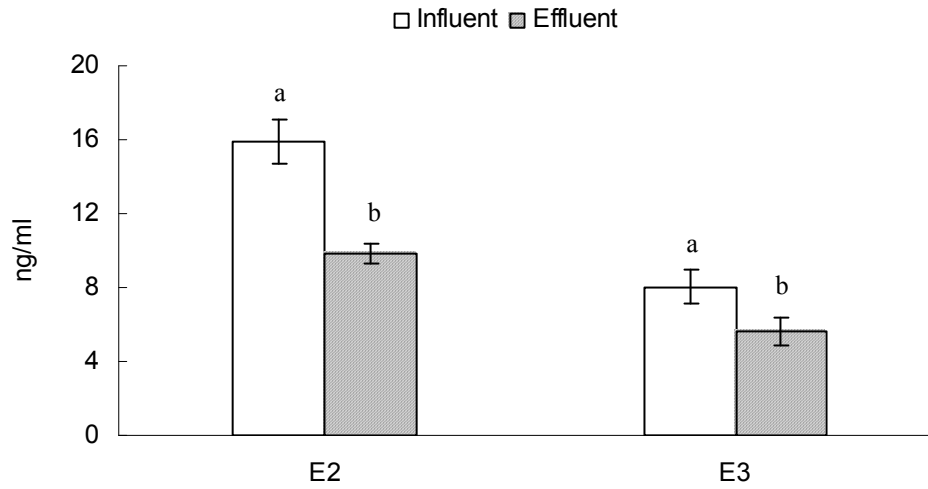


Figure 3.7 The location effect on E2 and E3 concentrations (ng/ml) in the Anaerobic Digester.

Data are reported as LSM \pm SE (n=9). The location effect was significant. There was no effect of interaction of season and sampling location. Bars with different letters are significantly different ($P < 0.05$).

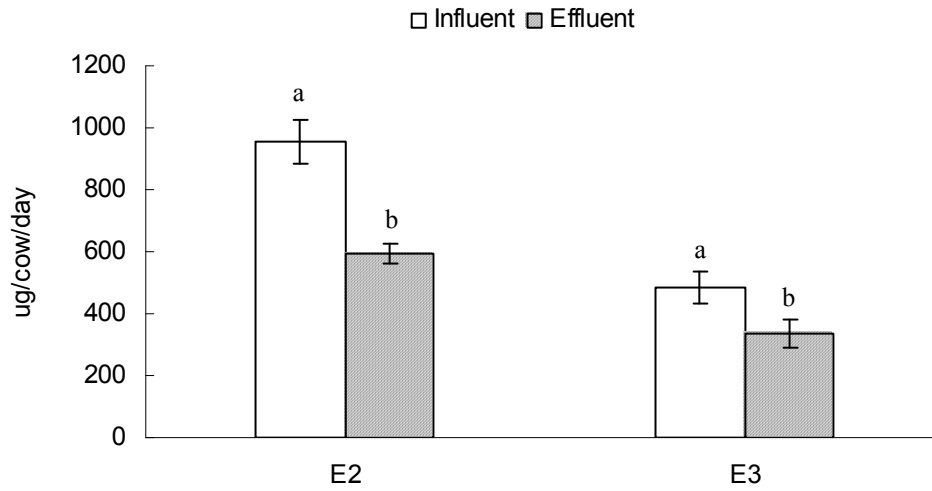


Figure 3.8 The location effect on E2 and E3 mass flow ($\mu\text{g}/\text{cow}/\text{day}$) in the Anaerobic Digester. Data are reported as $\text{LSM} \pm \text{SE}$ ($n=9$). The location effect was significant. There was no effect of interaction of season and sampling location. Bars with different letters are significantly different ($P < 0.05$).

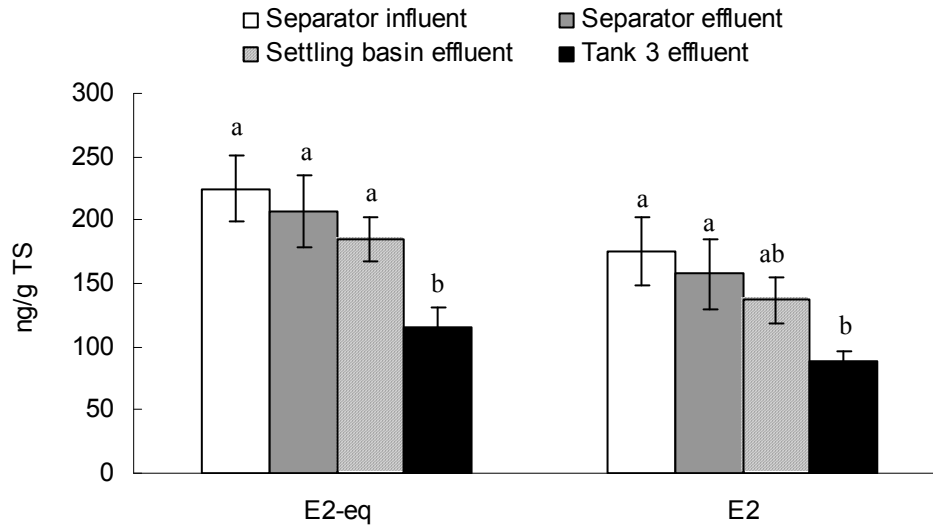


Figure 3.9 The concentrations of E2-eq and E2 (ng/g TS) at different sampling locations in the Separation/Aeration system. Data are reported as LSM \pm SE (n=3). The location effect was significant. Bars with different letters are significantly different ($P < 0.05$).

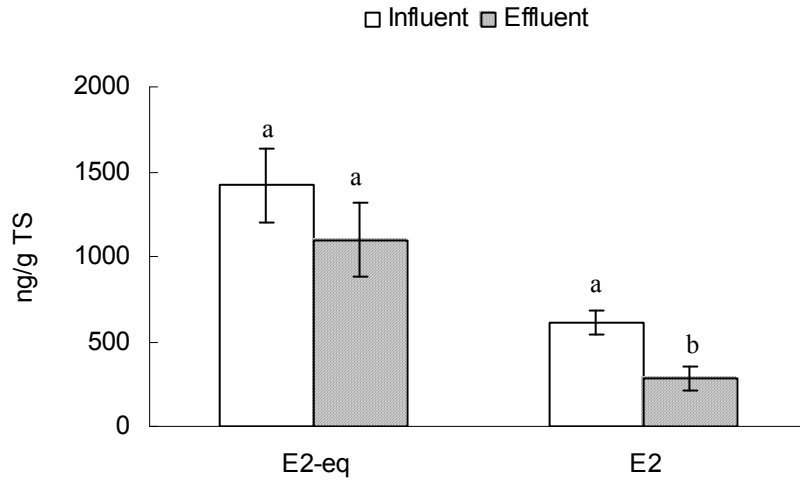


Figure 3.10 The concentrations of E2-eq and E2 (ng/g TS) in the influent and the effluent of the Anaerobic Digester. Data are reported as LSM \pm SE (n=3). The location effect was not significant for E2-eq. The location did affect E2. Bars with different letters are significantly different ($P < 0.05$).

Table 3.1 The contribution of E2 to total estrogenicity in the influent and effluent of different dairy manure treatment and storage systems.

System	N*	Influent		Effluent	
		Mean (%)	SD (%)	Mean (%)	SD (%)
Separation/Aeration	3	77	8	78	9
Anaerobic Digester	3	43	1	26	12
Conventional Storage	4	54	20	30	15

*N: three samplings for Separation/Aeration and Anaerobic Digester systems and four farms for Conventional Storage.

Table 3.2 The total solids (TS), E2, and E2-eq concentrations in barn manure and lagoon storages in four commercial swine farms.

Measure	Sample	Sow A ¹	Sow B ¹	Finishing C ²	Finishing D ²
TS (g/L)	Pit	220	141	94	21
	Primary lagoon	38	22	8	NA ³
	Secondary lagoon	NA	4.4	NA	1.3
E2 (ng/g TS)	Pit	2261	4188	218	1063
	Primary lagoon	1477	919	29	NA
	Secondary lagoon	NA	145	NA	34
E2-eq (ng/g TS)	Pit	3244	5073	650	2049
	Primary lagoon	2556	2030	67	NA
	Secondary lagoon	NA	252	NA	44
E2/E2-eq (%)	Pit	70	83	33	44
	Primary lagoon	58	45	43	NA
	Secondary lagoon	NA	58	NA	77

1. Sow A/B: farrowing to feeder/farrowing to wean farm
2. Finishing C/D: barrows and gilts farm
3. NA: not applicable.

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Chapter 4: Estrogen Removal from Dairy Manure by Pilot Scale Reactors

ABSTRACT

17 β -estradiol (E2) and total estrogenicity (E2-eq) removal from dairy manure was evaluated in six pilot scale reactors: two aerated reactors (60% and 100% aeration; AER60 and AER100), a nitrifying/denitrifying reactor (NDN), an enhanced biological phosphorus removal reactor (EBPR), an anaerobic digester (AD), and a nitrifying reactor (NIT) receiving AD effluent. The concentrations of E2 and E2-eq in all samples were determined with an enzyme-linked immunosorbent assay (ELISA) and yeast estrogen screen (YES) assay, respectively.

The influent had higher mass of E2 and E2-eq than the effluent in all reactors. The E2 and E2-eq removal efficiencies were expressed as % of the influent mass and also as %/aerobic hour of the influent mass because estrogens were degraded most in aerobic conditions. Estrogen removal in AD was expressed as % and %/hour of the influent mass to compare with aerobic reactors. Orthogonal contrasts were conducted to compare estrogen removal efficiencies based on reactor configurations or performances. Higher ammonia nitrogen removing reactors had higher E2 and E2-eq removal in %, higher E2 removal in %/aerobic hour, and the same E2-eq removal in %/aerobic hour compared to those with lower ammonia nitrogen removal. Estrogen removal efficiencies (both in % and %/aerobic hour) were similar in nitrifying and denitrifying reactors. Reactors with aeration supported greater estrogen removal than those without. Reactors with influent anaerobic digestion pretreatment resulted in the same E2 and E2-eq removal in % and higher E2 and E2-eq removal in %/aerobic hour compared to those without. Aeration appears to be necessary for more complete removal of estrogens from dairy manure.

Keywords: Estrogens, dairy manure, reactor, removal efficiency

INTRODUCTION

Various techniques including aeration, deammonification, enhanced biological phosphorus removal (EBPR), and anaerobic digestion are used in municipal wastewater treatment plants (WWTPs). These reactors are used to alter wastewater composition to meet a variety of environmental goals. They differ in hydraulic and solid retention time; inclusion, extent, and duration of aeration; and use and type of pretreatment. The effects of these systems on estrogen content of municipal wastes have been explored recently. With a conventional activated sludge system (aeration) in municipal treatment plants, 17 β -estradiol (E2) was reduced from 15.6 ng/L in the influent to 1.8 ng/L in the effluent (Servos et al., 2005). Natural estrogens were largely removed (>98%) in the denitrifying and nitrifying tanks in a municipal sewage treatment plant in Germany (Andersen et al., 2003). The effects of EBPR on estrogen removal from municipal wastes have not been reported. Carballa et al. (2007) observed 85% of removal of the sum of E2 and estrone (E1) through pilot scale conventional anaerobic digestion of sewage sludge.

Livestock wastes are very different in composition than municipal wastewater, with much higher solids content and very different pollutants of concern, limiting the applicability of results from WWTPs. Estrogen excretion by livestock in the United States was estimated at 49 tons annually (Lange et al., 2002), several-fold higher than was estimated for humans. Dairy cattle contribute the majority of these estrogens. It could be of significance to quantify the effects of manure treatments on estrogen content of dairy manure. Therefore, the objective of this study was to explore the effects of different types of pilot scale reactors designed to change nitrogen and phosphorus content in dairy manure (nitrifying, nitrifying/denitrifying, EBPR, and anaerobic digestion) on estrogen removal.

MATERIALS AND METHODS

Sample collection

Samples were collected from six pilot scale manure reactors. The basic reactor configuration data are listed in Table 4.1. Reactor I and Reactor II were aerated reactors constructed using 90 cm long and 20.3 cm of diameter PVC piping with a working volume of approximately 22 L (DeBusk et al., 2007). Liquid manure was collected after solid separation at the Virginia Tech Dairy Center to feed the reactors. The separator was a roller press, consisting of two perforated basins (concave screens with 0.79 mm screen opening), with a rotating brush assembly to convey slurry across the screens. Liquids flowed through the screen to a collection basin below the screens. Solids were advanced and forced out of the discharge by the rotating brushes. The mechanical separator used in this study was a commercially available unit (Integrity Nutrient Control System, Nutrient Control Systems, Inc., Chambersburg, PA). The reactor hydraulic retention time (HRT) was 3.33 days and was maintained using draw and fill operations that occurred four times per day. Reactor I and Reactor II were operated with continuous aeration (100%) and intermittent aeration (60%; 1h: 0.67h), respectively, to conserve nitrogen through nitrification. The influent air flow rate was 2.9 L/min. The influent and effluent samples were collected once a week for three consecutive weeks. The two reactors were denoted AER60 and AER100, respectively.

Reactor III included a manure storage tank, a completely mixed pilot scale fermenter, and a nitrification/denitrification reactor (150 L; HRT = 10 days) in series. Its purpose was to remove bioavailable ammonia nitrogen. Scraped manure from the Virginia Tech Dairy Center was diluted with tap water and separated through a research scale version of the above described mechanical manure separator (3.18 mm screen opening) before being fed to the fermenter. The

reactor was operated on an eight-hour cycle, which started with 5 L fermented manure pumped in. This was followed by a 70 minute anoxic phase, a 310 minute nitrification phase (aerobic zone), and another 100 minute anoxic phase. Samples from the manure storage tank, the fermenter, and the reactor after each of the three phases (daytime cycle only) were collected once every other day (n=3). This reactor was denoted NDN.

Reactor IV was an enhanced biological phosphorus removal reactor (EBPR). It included a manure storage tank, a fermenter, and a sequencing batch reactor (SBR; HRT = 2 days; SRT = 4 days). Phosphate accumulating organisms (PAOs) in the SBR transform VFAs, the fermented end product from the fermenter, into biopolymers under anaerobic conditions (Grady et al., 1999). PAOs then uptake phosphorus by using the energy stored in the biopolymers under aerobic conditions where oxygen was supplied with aeration. At the end of each treatment cycle, phosphorus deficient liquid (effluent) was discharged from the system. Furthermore, phosphorus rich biomass mixed liquor was wasted from the SBR. This system was operated on an eight-hour cycle, which started with a 40 minute anaerobic phase (air off) and a 70 minute aerobic phase (air on), repeated three times across the eight hours. 4.8 L liquid manure (scraped manure from Virginia Tech Dairy Center as Reactor III diluted with tap water and separated) was pumped into the SBR with 1.2 L at the beginning of each anaerobic phase. At the end of each cycle, 3.6 L nutrient depleted effluent and 1.2 L wasted mixed liquor and suspended solids (MLSS) were discharged from the system. The feed, fermenter, effluent, and MLSS were sampled once every other day (n=3).

Reactor V was an anaerobic digester with 15 L of volume and 5 days of HRT. Liquid manure after full-scale solid separation in the Virginia Tech Dairy Center was collected to 'feed' the anaerobic digester as in Reactor I and II. It was operated on a four-hour cycle, with filling of

0.5 L of liquid manure and drawing of 0.5 L of anaerobically digested effluent. The temperature in the anaerobic digester was maintained at 35.2 °C. Samples of the liquid manure influent and the anaerobic digester effluent were collected once every other day (n=3). The reactor was denoted AD.

Reactor VI was a nitrification reactor with 12 L of volume and 4 days of HRT. This reactor was fully aerated to treat anaerobically digested dairy manure effluent of Reactor V (AD). It was operated on a four-hour cycle, with filling of 0.5 L of anaerobically digested dairy manure and drawing of 0.5 L of nitrified effluent. Samples of the influent and effluent were collected once every other day (n=3). The reactor was denoted NIT.

Reactor dissolved oxygen (DO) in all reactors and total solids (TS) concentrations of all samples were determined according to standard methods (APHA, 1998). All samples were processed on the day of sampling.

Sample extraction

Samples were extracted using the method described by Zhao et al. (2008). Briefly, one ml of homogenized (magnetic stir plate) liquid manure sample was thoroughly mixed with 1.5 ml of chloroform and 1.5 ml of NaOH (1 M) in 12 * 75 mm glass tubes (Corning Life Sciences, Lowell, MA) and centrifuged for 20 minutes at 2500 x g. One ml of the supernatant alkaline phase was removed and neutralized with 180 µl of 90% acetic acid, and was further extracted with 3 ml of toluene twice. The combined 6 ml of toluene was then evaporated to dryness with a gentle nitrogen gas stream and stored at -20 °C for final assays.

Estrogen analysis

The concentrations of E2 in all samples were assayed with a commercial enzyme-linked immunosorbent assay kit (Zhao et al., 2008). The kit uses a polyclonal antibody to bind, in a

competitive manner, free estrogens in the standards and samples, or enzyme labeled estrogens contained in the kit. The dried sample extract was resuspended with 300-1000 μL of an assay buffer solution contained in the kit and a 100 μL aliquot was added to the wells in duplicates together with 50 μL of enzyme-labeled E2 and antibodies. After a simultaneous incubation (2 hours) at room temperature on a plate shaker (500 rpm) the excess reagents were washed away and the substrate for the enzyme was added for color development. After another incubation (45 minutes) without shaking the color reaction was stopped and the color change was quantified by measuring absorbance at 405 nm wavelength (MRX Revelation 4.22, Dynex Technology Inc, Chantilly, VA). Every plate contained six E2 standards, ranging from 29 to 30,000 pg/ml in duplicated wells from which standard curves were developed.

The concentrations of total estrogenicity expressed as E2 equivalent (E2-eq) in all samples were determined using yeast estrogen screen (YES) assay as described by Zhao et al. (2008). This assay consists of an estrogen-inducible expression system in a recombinant yeast strain (*Saccharomyces cerevisiae*). The human estrogen receptor gene was integrated into the yeast genome, which also contains expression plasmids carrying estrogen-responsive sequences controlling expression of the reporter gene *lac-Z*. Upon binding of active ligands to the human estrogen receptor, *lac-Z* is expressed to produce the enzyme β -galactosidase, which is secreted into the medium. It then metabolizes the chromogenic substrate, chlorophenol red- β -D-galactopyranoside (CPRG) (Roche Diagnostics, Indianapolis, IN), from yellow into a red product.

All operations were conducted in a laminar fume hood (Contamination Control Inc, Lansdale, PA). In the 96-well sterile flat-bottom microtiter plates (Cat. 353072, BD Biosciences, San Jose, CA), 100 μL of sample extract resuspended with absolute ethanol (500-1000 μL) was added to wells for serial dilution across rows and 10 μL from each dilution was transferred to

blank wells in triplicates. After being evaporated to dryness in air, 200 µl of growth medium containing yeast cells (grown to absorbance of 1 at 600 nm) was added to each well (Holbrook et al., 2002). The plates were incubated at 32 °C for 3 days and then 2 more days at room temperature. The color density was quantified by measuring absorbance at 575 nm (the highest absorption) and 620 nm (background) (Spectracount Microplate Photometer BS-10,000, Packard, Meriden, CT). Every plate contained two rows of E2 standard (>98% purity, Sigma Chemical Company, St. Louis, MO), ranging from 7.8-1000 pg/ml from which a standard curve was developed.

Estrogen mass flow and removal

Estrogen mass flow of the six reactors was calculated based on estrogen concentrations and feeding and discharging volume. The fermenter that was used to pretreat manure before reactors (NDN and EBPR) did not alter E2 or E2-eq concentrations significantly (data not shown); therefore, E2 and E2-eq concentrations in feed tank were used as reactor influent values.

Estrogen removal in each cycle was the difference of estrogen mass in the influent and the effluent of the same volume. Aerobic hour length in each cycle (the period when reactors were aerated) was also considered to calculate estrogen removal efficiency per aerobic hour. Estrogen removal efficiency per hour was calculated in the anaerobic digester.

Statistical analysis

The effects within each of the six reactors on estrogen mass flow were analyzed using the GLM procedure of SAS (SAS Institute, 2004) according to the following model:

$$Y_{ij} = \mu + L_i + e_{ij},$$

where

μ = overall mean,

L_i = location effects ($i = 1, 2$),

e_{ij} = residual error ($j = 1, 2, 3$).

There were two sampling locations (influent and effluent) with three sampling replicates for each reactor. Data were reported as least square means (LSM) \pm standard error (SE). LSM were tested with one-way ANOVA and differences were declared significant at $P < 0.05$.

The effects of the six reactors on E2 and E2-eq removal efficiencies were compared using the GLM procedure of SAS (SAS Institute, 2004) according to the following model:

$$Y_{ij} = \mu + R_i + e_{ij},$$

where

μ = overall mean,

R_i = reactor effects ($i = 1, 2, 3, 4, 5, 6$),

e_{ij} = residual error ($j = 1, 2, 3$).

Data were reported as LSM \pm SE. Orthogonal contrasts based on different reactor configurations (nitrifying vs. denitrifying, aerobic vs. anaerobic, and influent treatment vs. no treatment) or performances (high vs. low ammonia nitrogen removal) were used to compare estrogen removal efficiencies ($P < 0.05$).

RESULTS AND DISCUSSION

Estrogen mass flow and removal within each reactor

The influent and effluent mass of E2-eq and E2 within each reactor is shown in Figure 4.1 and Figure 4.2. AER60 had significantly higher E2-eq (3464 vs. 916 ng) and E2 (847 vs. 377 ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 74% and 55%, respectively. 21% of E2-eq and 15% of E2 were removed per aerobic hour by AER60 (3.6 aerobic hours/cycle). Similarly, AER100 had significantly higher E2-eq (3464 vs. 359 ng) and

E2 (847 vs. 109 ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 90% and 87%, respectively. 15% of E2-eq and E2 were removed per aerobic hour by AER100 (6 aerobic hours/cycle). Others have reported estrogen removal in nitrification process as with AER60 and AER100 (Shi et al., 2004b; Ren et al., 2007).

NDN had significantly higher E2-eq (5990 vs. 120 ng) and E2 (4945 vs. 363 ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 98% and 92%, respectively. 19% of E2-eq and 18% of E2 were removed per aerobic hour (5.2 aerobic hours/cycle). These efficiencies were comparable to those reported in WWTPs. The total mass of E1 and E2 was removed by >98% in denitrifying and nitrifying tanks in a municipal wastewater treatment plant (Andersen et al., 2003). Greater than 95% of total estrogenicity was removed by activated sludge treatment plus nitrification/denitrification in a sewage treatment plant (Leusch et al., 2005).

EBPR had significantly higher E2-eq (6622 vs. 874 ng) and E2 (4141 vs. 925 ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 87% and 77%, respectively. 19% of E2-eq and 16% of E2 were removed per aerobic hour (4.7 aerobic hours/cycle). EBPR is operated on sequencing anaerobic and aerobic conditions and estrogen degradation in both conditions has been widely reported. The only study reported regarding estrogen removal from livestock manure by EBPR was a conference abstract (Isabelle et al., 2007), in which E2-eq and E1 were reported to be reduced by 36% and 68%, respectively, by an EBPR treating swine manure. The reactor configuration data were not available. E2 was not detected in swine manure influent of EBPR after aerobic thermophilic SBR (Isabelle et al., 2007). In that study, *Mycobacterium* sp., *Methylobacterium* sp., *Ochrobactrum* sp., and *Pseudomonas* sp. were cultured on the activated sludge, isolated, and identified as E1, E2, and equol degraders.

Interestingly, *Methylobacterium* sp. even converted E1 to E2 at a rate of 80% in 96 hours, which resulted in increased total estrogenicity; the conversion of E2 from E1 was rarely reported.

NIT had significantly higher E2-eq (563 vs. 76 ng) and E2 (121 vs. 8 ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 87% and 93%, respectively. The removal efficiencies were 22% (E2-eq) and 23% (E2) per aerobic hour (4 aerobic hours/cycle).

AD had significantly higher E2-eq (862 vs. 563 ng) and E2 (324 vs. 121ng) mass in the influent than in the effluent. The E2-eq and E2 removal efficiencies were 35% and 62%, respectively. E2-eq and E2 were removed 9% and 15%, respectively, per hour (4 hours/cycle). Higher removal efficiency of E2-eq (80%) was reported in a thermophilic anaerobic digester treating dairy manure with higher temperature (56 °C) and longer HRT (19 days) (Ermawati et al., 2007). However, Carballa et al. (2007) did not observe significant effects of temperature (37 vs. 55 °C) on removal of the sum of E1 and E2 by a pilot scale anaerobic digester treating sewage sludge.

Estrogen removal efficiency comparison

The mass removal efficiencies of E2-eq and E2 in % and in %/aerobic hour (or hour) were shown in Figure 4.3, Figure 4.4, Figure 4.5, and Figure 4.6. There were significant differences in both E2-eq and E2 removal efficiencies among reactors.

High vs. low ammonia nitrogen removal

AER100, NDN, and NIT had high removal rates of ammonia nitrogen (average 98%). AER60 only removed 22% of influent ammonia nitrogen. The ammonia nitrogen removal data were not available with EBPR. Therefore, the average of AER100, NDN, and NIT was contrasted to AER60 (high vs. low ammonia nitrogen removal).

High ammonia nitrogen removing reactors had higher E2 and E2-eq removal in % than those with low ammonia nitrogen removal. Estrogen degradation has been reported during nitrification (Vader et al., 2000; Shi et al., 2004a; Ren et al., 2007; Shi et al., 2007; Yi and Harper, 2007) as AER100, NIT, and NDN all had nitrifying process. Vader et al. (2000) reported degradation of 17 α -ethinylestradiol (EE2) in nitrifying activated sludge (NAS), but no degradation was detected with a much lower ammonia oxidizing rate. Ren et al. (2007) observed lower estrogen degradation associated with lower ammonia oxidation in NAS. The co-metabolism of ammonia oxidizing bacterium (AOB) dominated the degradation of E1, E2, and EE2 in NAS (Shi et al., 2004a). A linear relationship ($R^2 = 0.94$) was found between ammonia biotransformation rate and EE2 biotransformation rate by enriched nitrifying cultures (Yi and Harper, 2007).

The different total ammonia nitrogen removal rates (98 vs. 22%) explained the significant differences in E2 and E2-eq removal efficiencies in % between high and low ammonia nitrogen removing reactors, although the ammonia stripping loss was not accounted for. The ammonia monooxygenase from AOB, such as *Nitrosomonas europaea*, is able to degrade estrogens and other hydrocarbons (Keener and Arp, 1994; Chang et al., 2002; Shi et al., 2004b). Most AOB were related to *Nitrosomonas* observed in activated sludge and full-scale WWTPs (Mobarry et al., 1996; Purkhold et al., 2000). NAS degraded natural estrogens and EE2 and their intermediates, but *Nitrosomonas europaea* only degraded estrogens without further degradation (Shi et al., 2004a). This indicates other microorganisms in NAS are responsible for estrogen intermediate degradation. Yoshimoto et al. (2004) reported *Rhodococcus zopfii* and *R. equi* isolates from activated sludge can also degrade estrogens.

High ammonia nitrogen removing reactors had higher E2 removal in %/aerobic hour and the same E2-eq removal in %/aerobic hour compared to those with low ammonia nitrogen removal. The low ammonia nitrogen concentration in AER100, NDN, and NIT (data not shown) may have actually limited the community of AOB, as indicated by the insignificantly different E2-eq removal efficiencies (%/aerobic hour) from that of AER60. Greater loss of ammonia oxidizing activity (85 vs. 35%) in *Nitrosomonas europaea* was observed after 24 hour incubation in growth medium containing limited ammonium (15 vs. 50 mM ammonium) (Stein and Arp, 1998). The decay rate and maintenance energy demand of *Nitrosomonas europaea* were increased with ammonia depletion (Geets et al., 2006). The higher E2 removal efficiency in %/aerobic hour of high ammonia nitrogen removing reactors deserves more research.

Nitrifying vs. nitrifying/denitrifying

AER100 and NIT were both nitrifying reactors. NDN had both nitrifying and denitrifying processes. Therefore, the average of AER100 and NIT was contrasted with NDN (nitrifying vs. nitrifying/denitrifying). There were no significant differences of E2-eq and E2 removal efficiencies in % and %/aerobic hour between nitrifying and nitrifying/denitrifying reactors.

The mechanism for estrogen removal in the aerobic phase of NDN was the same as the two nitrifying reactors AER100 and NIT. Anoxic degradation of estrogens or hydrocarbons is also possible. Czajka and Londry (2006) reported 180 µg/L/day of E2 degradation rate in nitrate-reducing culture (anoxic) established with lake water and sediment for the first 14 days with an original concentration of 5 mg/L, but this was even lower than in the anaerobic condition (260 µg/L/day). Dimethyl phthalate was degraded in denitrifying process as an electron donor (Liang et al., 2007). Most ammonia nitrogen was removed in nitrifying (average 97%) and nitrifying/denitrifying (100%) reactors. The anoxic phases of NDN did not remove E2-eq or E2

significantly (data not shown). This explained the insignificant difference of estrogen removal efficiency between nitrifying and nitrifying/denitrifying reactors.

Aerobic vs. anaerobic

AER60, AER100, NDN, NIT, and EBPR were aerobic or aerobic condition dominant reactors. AD was an anaerobic digester treating dairy manure. Therefore, the average of AER60, AER100, NDN, NIT, and EBPR was contrasted to AD (aerobic vs. anaerobic). The aerobic reactors removed E2-*eq* and E2 more efficiently both in % and in %/aerobic hour than the anaerobic reactor (% and %/hour).

More estrogen degradation in aerobic conditions than in anaerobic conditions has been widely reported (Lee and Liu, 2002; Joss et al., 2004; Lorenzen et al., 2004). The different estrogen degradation rates are due to different estrogen degradation pathways in aerobic and anaerobic conditions. In aerobic conditions, the introduction of hydroxyl groups by mono- and dioxygenases, carboxylation, ring cleavage, and final decarboxylation are the basic steps for degradation of phenolic compounds (Rehm et al., 2001; Seo et al., 2007). Anaerobes may degrade phenolic compounds through hydroxylation and carboxylation as the initial activation steps (Chakraborty and Coates, 2005; Heider, 2007). The aromatic rings need to be reduced to destabilize the resonant electron distribution. Then the ring structure is broken through reductive cleavage to form aliphatic acids to produce metabolites suitable for methanogenic use (Evans, 1977; Grbic-Galic and Vogel, 1987).

Influent treatment vs. no treatment

NIT and AER100 were both fully aerated reactors with high DO concentrations. The major difference was that the influent of NIT was anaerobically digested. Therefore, NIT and AER100 were contrasted (influent treatment vs. no treatment). NIT and AER100 had no

significant differences in E2 and E2-eq removal efficiency in %, despite that NIT influent had lower E2 and E2-eq concentrations and E2 ratio to E2-eq than AER100 influent as a result of anaerobic digestion pretreatment. The same ammonia nitrogen removal rates explained the insignificant difference of E2 and E2-eq removal efficiencies in % between NIT and AER100 as discussed in the contrast of high and low ammonia nitrogen removal.

The longer aerobic hours of AER100 resulted in lower E2 and E2-eq removal efficiencies (%/aerobic hour) than NIT, which is self-explanatory because the percent removal of E2 and E2-eq was the same. This means estrogen degradation slowed down as aeration cycle advanced, with a plateau observed at 6 h on cycle of AER100.

E2 and E2-eq removal comparison

All aerobic reactors except AER60 had the same removal efficiency for E2 and E2-eq (Figure 4.2 and 4.3). Natural estrogens (E1, E2, and E3) account for most of E2-eq in livestock manure (Furuichi et al., 2006; Shappell et al., 2007). Andersen et al. (2004) reported higher aerobic degradation rate of E2 than that of E1 in activated sewage sludge. Shi et al. (2004a) also found faster E2 degradation than E1 and E3 by NAS. Therefore, the reasonable explanation of the same removal rates of E2 and E2-eq was that both of them were degraded essentially completely in the aerobic reactors. The lower E2 removal efficiency than that of E2-eq in AER60 deserves further research.

Interestingly, AD removed E2 more efficiently than E2-eq (62 vs. 35%). The full-scale anaerobic digester on the large commercial dairy farm (Chapter 3) also removed E2 more efficiently than E2-eq (54 vs. 23%). E2 was degraded faster than E1 under anaerobic conditions in activated sludge (Andersen et al., 2004). Based on recalculation from a study on swine manure treated by up-flow anaerobic sludge blanket (essentially anaerobic digester) (Furuichi et al.,

2006), E2 was removed the most (63%) compared to other natural estrogens E1 (30%), E3 (21%), and 17 α -estradiol (6%). This explained why E2 was removed more efficiently than E2-eq in the anaerobic digester treating dairy manure. In addition to that, the release and/or formation of new estrogenic compounds in anaerobic digestion may help explain the differences in E2 and E2-eq removal efficiencies.

All the aerobic reactors except NIT had higher ratios of E2 to E2-eq in the effluent than in the influent (data not shown). In contrast, AD had lower ratios of E2 to E2-eq in the effluent than in the influent (22 vs. 37%). The authors also found the same decrease of E2 contribution to E2-eq in a full-scale anaerobic digester treating dairy manure on a large commercial dairy farm (43 vs. 26%; Chapter 3). Furuichi et al. (2006) also reported decreased ratios of E2 to E2-eq (27 vs. 16%) in swine manure after anaerobic digestion. This change corresponded to the different removal rates of E2 and other estrogenic compounds in livestock manure.

CONCLUSIONS

All reactors substantially removed the mass of E2 and E2-eq in the influent. The E2 and E2-eq removal efficiencies were significantly different, with 55-93% of E2 and 35-98% of E2-eq removed. The removal efficiency per aerobic hour or per hour ranged from 15 to 23% (E2) and 9 to 22% (E2-eq).

Ammonia nitrogen oxidation and aeration appeared to be the key factors influencing estrogen removal, as indicated by higher E2 and E2-eq removal (%) with higher ammonia nitrogen oxidation or aerated (aerobic) reactors than those with lower ammonia nitrogen oxidation or without aeration (anaerobic). This corresponds to the dominated degradation of estrogens by co-metabolism of AOB or different estrogen degradation pathways in aerobic and anaerobic conditions. The additional denitrification did not support more estrogen degradation

than those with nitrification only, which is in contrast to the reported estrogen degradation in denitrification conditions by other studies. E2 and E2-eq removal was also faster (%/aerobic hour) associated with higher ammonia nitrogen removal or aeration, except that the E2-eq removal rates were not affected by ammonia nitrogen removal rates. The anaerobic reactor removed E2 more efficiently than E2-eq in contrast to the same E2 and E2-eq removal efficiencies in most aerobic reactors, which reflected different estrogen degradation rates or possibly the release or formation of new estrogenic compounds in anaerobic conditions. Future research will focus on estrogen removal mechanisms and kinetics with different reactor configurations and performances.

Table 4.1 The characteristics of the six dairy manure reactors.

	AER60 ^a	AER100 ^b	NDN ^c	EBPR ^d	AD ^e	NIT ^f
Reactor volume (L)	16	16	150	28.8	15	12
Hydraulic retention time (day)	3.33	3.33	10	2	5	4
Solid retention time (day)	3.33	3.33	10	4	5	4
Cycle length (hour)	6.0	6.0	8.0	8.0	4.0	4.0
Aerobic phase (hour)	3.6	6.0	5.2	4.7	NA ^g	4.0
Aerobic phase (%)	60	100	65	59	NA	100
Fill/Draw volume (L)	1.2	1.2	5	4.8	0.5	0.5
Dissolved oxygen (mg/L)	0.7/4.45 ^h	4.4	0/1.3 ^h	0.14/4 ^h	NA	4.8
Total solids (g/L)	15.5	14.5	15.3	18.5	9.1	8.8

- a. AER60: 60% aeration
- b. AER100: 100% aeration
- c. NDN: nitrification/denitrification reactor
- d. EBPR: enhanced biological phosphorus removal
- e. AD: anaerobic digester
- f. NIT: nitrification receiving AD effluent
- g. NA: not applicable
- h. Dissolved oxygen: air off/air on

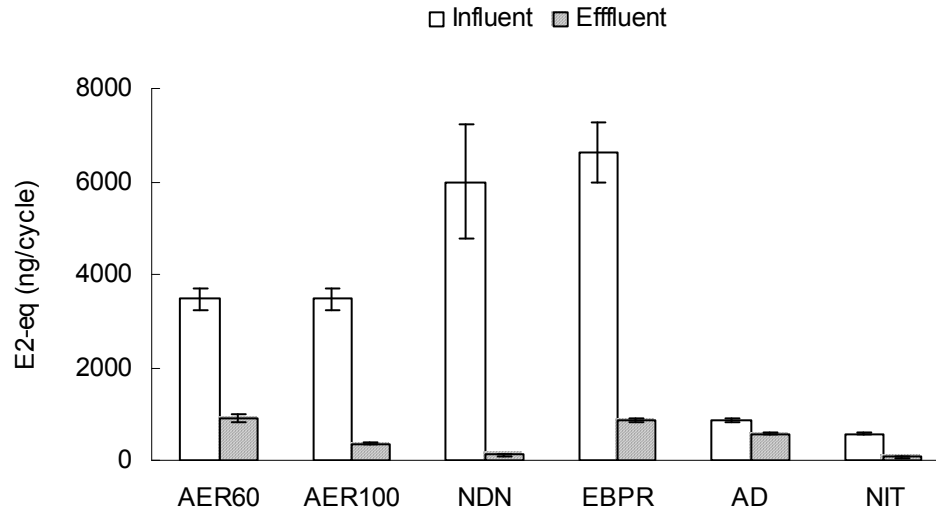


Figure 4.1 The mass flow of E2-eq per cycle in different pilot scale reactors. Data are reported as LSM \pm SE (n = 3). The influent had significantly more E2-eq mass than the effluent in all reactors ($P < 0.05$). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent.

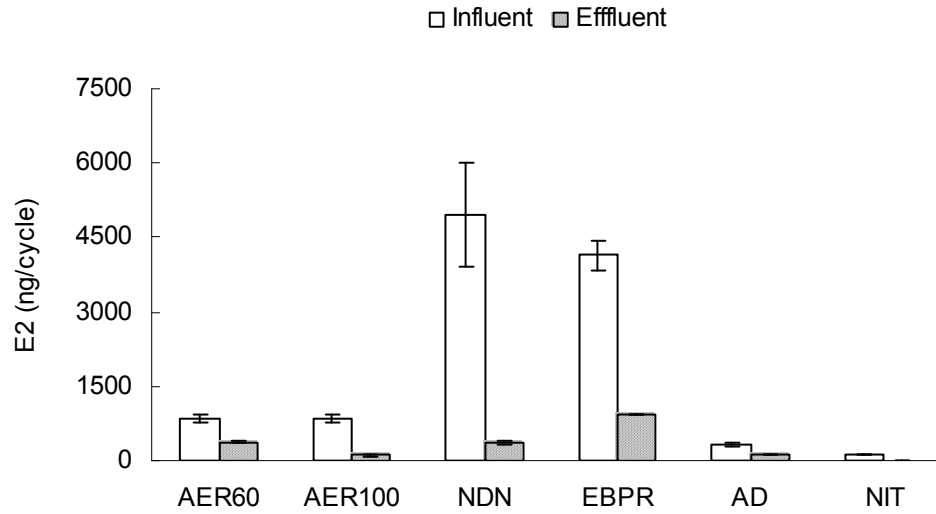


Figure 4.2 The mass flow of E2 per cycle in different pilot scale reactors. Data are reported as LSM \pm SE (n = 3). The influent had significantly more E2 mass than the effluent in all reactors ($P < 0.05$). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent.

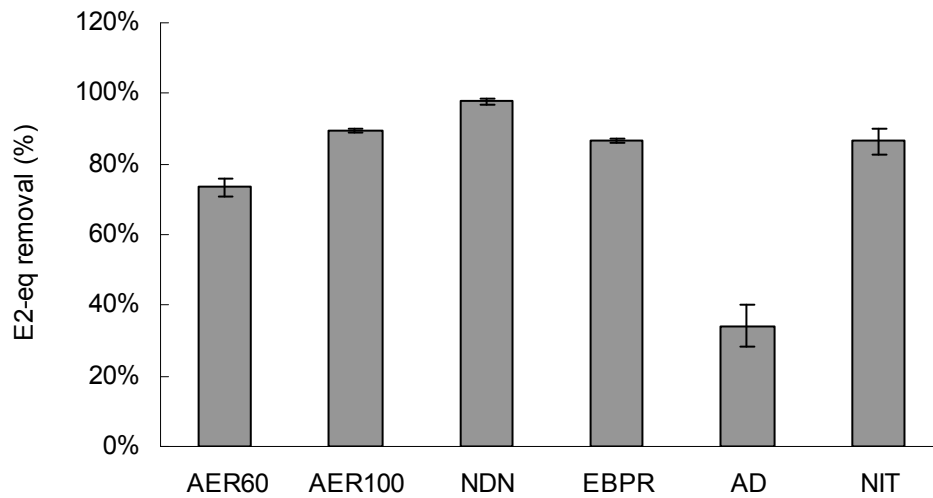


Figure 4.3 The E2-eq mass removal efficiency (%) in different pilot scale reactors. Data are reported as LSM \pm SE ($n = 3$). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent. Higher ammonia nitrogen removal (the average of AER100, NIT, and NDN vs. AER60, $P < 0.0005$) or aeration (the average of the other five vs. AD, $P < 0.0001$) resulted in higher E2-eq removal than those with lower ammonia nitrogen removal or without aeration, respectively. Reactors with denitrification (NDN vs. the average of AER100 and NIT, $P < 0.0590$) or influent anaerobic digestion pretreatment (NIT vs. AER100, $P < 0.4909$) were similar in E2-eq removal as those without influent pretreatment or denitrification.

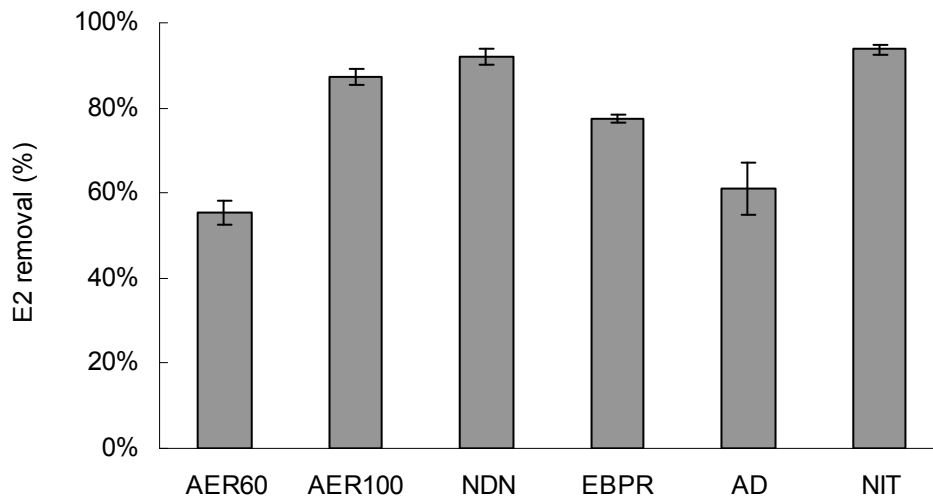


Figure 4.4 The E2 mass removal efficiency (%) in different pilot scale reactors. Data are expressed as LSM \pm SE (n = 3). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent. Higher ammonia nitrogen removal (the average of AER100, NIT, and NDN vs. AER60, $P < 0.0001$) or aeration (the average of the other five vs. AD, $P < 0.0001$) resulted in higher E2 removal than those with lower ammonia nitrogen removal or without aeration, respectively. Reactors with denitrification (NDN vs. the average of AER100 and NIT, $P < 0.7237$) or influent anaerobic digestion pretreatment (NIT vs. AER100, $P < 0.1713$) were similar in E2 removal as those without denitrification or influent pretreatment.

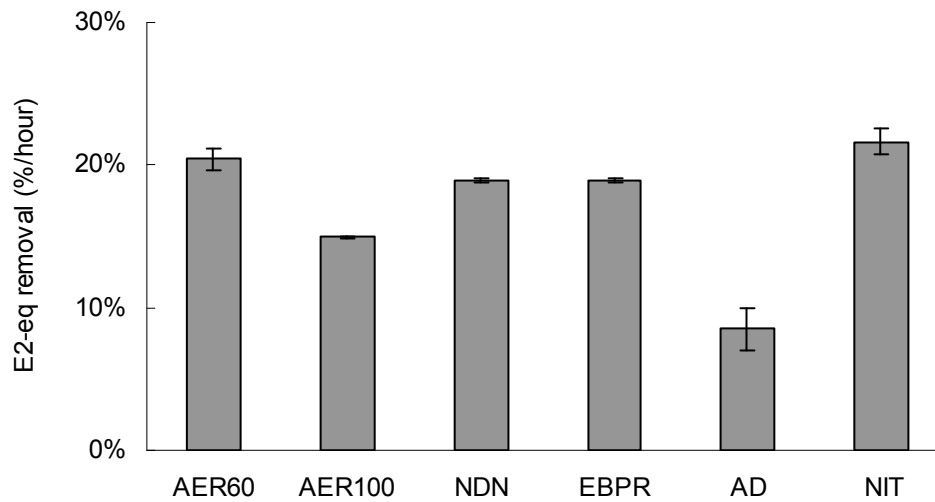


Figure 4.5 The E2-eq mass removal efficiency per aerobic hour or per hour (by AD only) in different pilot scale reactors. Data are reported as LSM \pm SE ($n = 3$). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent. Reactors with higher ammonia nitrogen removal (the average of AER100, NIT, and NDN vs. AER60, $P < 0.0617$) or denitrification (NDN vs. the average of AER100 and NIT, $P < 0.5317$) were similar in E2-eq removal as those with lower ammonia nitrogen removal or without denitrification, respectively. Reactors with aeration (the average of the other five vs. AD, $P < 0.0001$) or influent anaerobic digestion pretreatment (NIT vs. AER100, $P < 0.0001$) resulted in higher E2-eq removal than those without aeration or influent pretreatment, respectively.

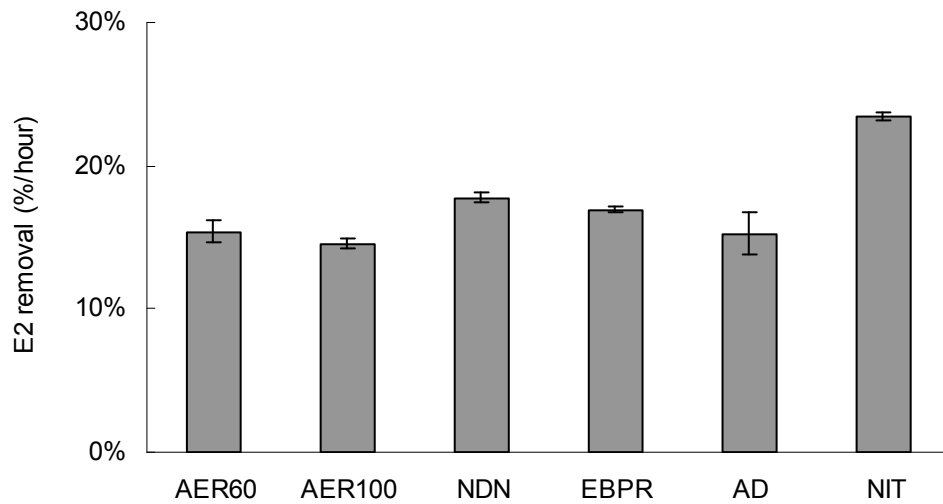


Figure 4.6 The E2 mass removal efficiency per aerobic hour or per hour (by AD only) in different pilot scale reactors. Data are reported as LSM \pm SE ($n = 3$). AER60: 60% aeration; AER100: 100% aeration; NDN: nitrification/denitrification; EBPR: enhanced biological phosphorus removal; AD: anaerobic digester; NIT: nitrification receiving AD effluent. Reactors with denitrification (NDN vs. the average of AER100 and NIT, $P < 0.2144$) were similar in E2 removal as those without denitrification. Reactors with higher ammonia nitrogen removal (the average of AER100, NIT, and NDN vs. AER60, $P < 0.0036$), aeration (the average of the other five vs. AD, $P < 0.0163$), or influent anaerobic digestion pretreatment (NIT vs. AER100, $P < 0.0001$) resulted in higher E2 removal than those with lower ammonia nitrogen removal, without aeration, or without influent pretreatment, respectively.

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APPENDIX

THE PROTOCOL OF 17 β -ESTRADIOL ELISA (ASSAY DESIGNS)

Materials supplied

1. Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells

A plate using break-apart strips coated with goat antibody specific to rabbit IgG.

2. 17 β -estradiol EIA Conjugate, 6 mL

A blue solution of alkaline phosphatase conjugated with 17 β -Estradiol.

3. 17 β -estradiol EIA Antibody, 6 mL

A yellow solution of a rabbit polyclonal antibody to 17 β -Estradiol.

4. Assay Buffer 3, 30 mL

Tris buffered saline containing proteins and detergents and sodium azide as a preservative.

5. Wash Buffer Concentrate, 30 mL

Tris buffered saline containing detergents.

6. 17 β -estradiol Standard, 0.5 mL

A solution of 300,000 pg/mL 17 β -estradiol.

7. Steroid Displacement Reagent, 1 mL

A special formulated displacer to inhibit steroid binding to proteins.

8. pNpp Substrate, 20 mL

A solution of p-nitrophenyl phosphate in buffer. Ready to use.

9. Stop Solution, 5 mL

A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic.

10. 17 β -estradiol Assay Layout Sheet, 1 each

11. Plate Sealer, 1 each

Storage

All components of this kit are stable at 4 °C until the kit's expiration date.

Materials needed but not supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 μ L and 1,000 μ L.
3. Repeater pipets for dispensing 50 μ L and 200 μ L.
4. Disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Procedural notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. Care must be taken to minimize contamination by endogenous alkaline phosphatase.

Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.

9. Prior to addition of substrate, ensure that there is no residual wash buffer in wells. Any remaining wash buffer may cause variation in assay results.

Reagent preparation

17 β -estradiol standard

Allow the 300,000 pg/mL 17 β -estradiol standard solution to warm to room temperature. Label six 12 x 75 mm glass tubes #1 through #6. Pipet 1,000 μ L of standard diluent (Assay Buffer 3) into tube #1. Pipet 750 μ L of standard diluent into tubes #2 through #6. Remove 100 μ L of diluent from tube #1. Add 100 μ L of the 300,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Add 250 μ L of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #6.

The concentration of 17 β -estradiol in tubes #1 through #6 will be 30,000, 7,500, 1,875, 468.8, 117.2, and 29.3 pg/mL, respectively. See the 17 β -estradiol Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

Wash buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
 2. Pipet 100 µL of standard diluent (Assay Buffer 3) into the NSB and the Bo (0 pg/mL Standard) wells.
 3. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.
 4. Pipet 100 µL of the Samples into the appropriate wells.
 5. Pipet 50 µL of Assay Buffer 3 into the NSB wells.
 6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
 7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.
- NOTE:** Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.
8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
 9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
 10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
 11. Add 5 µL of the blue Conjugate to the TA wells.

12. Add 200 μL of the p-Npp Substrate solution to every well. Incubate at room temperature for 45 minutes without shaking.

13. Add 50 μL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of results

Several options are available for the calculation of the concentration of 17β -estradiol in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of 17β -estradiol can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (B_0), using the following formula:

$$\text{Percent Bound} = \text{Net OD} / \text{Net } B_0 \text{ OD} \times 100$$

3. Using Logit-Log paper plot Percent Bound versus Concentration of 17β -estradiol for the standards. Approximate a straight line through the points. The concentration of 17β -estradiol in the unknowns can be determined by interpolation.

THE PROTOCOL OF YEAST SCREEN ASSAY

Preparation of minimal medium and medium components

Minimal medium and medium components prepared in glassware contaminated with an oestrogenic chemical will lead to elevated background expression. Glassware, spatulas, stirring bars, etc., must be scrupulously cleaned, and should not have had prior contact with steroids.

Rinse glassware, spatulas, stirring bars twice with absolute ethanol, and leave to dry.

Alternatively, wash twice with methanol, and once with ethanol.

Minimal Medium (pH 7.1)

Add 13.61 g KH_2PO_4 , 1.98 g $(\text{NH}_4)_2\text{SO}_4$, 4.2 g KOH pellets, 0.2 g MgSO_4 , 1 ml $\text{Fe}_2(\text{SO}_4)_3$ solution (40 mg/50 ml H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 L double-distilled water. Place on heated stirrer to dissolve. Dispense 45 ml aliquots into glass bottles.

Sterilize at 121°C for 10 min, and store at room temperature.

D-(+)-Glucose

Prepare a 20% w/v solution.

Sterilize in 20 ml aliquots at 121°C for 10 min.

Store at room temperature.

L-Aspartic Acid

Make a stock solution of 4 mg/ml.

Sterilize in 20 ml aliquots at 121°C for 10 min.

Store at room temperature.

Vitamin Solution

Add 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml H₂O) to 180 ml double-distilled water.

Sterilize by filtering through a 0.2- μ m pore size disposable filter, in a laminar air flow cabinet.

Filter into sterile glass bottles in 10 ml aliquots.

Store at 4°C.

L- Threonine

Prepare a solution of 24 mg/ml.

Sterilize in 10 ml aliquots at 121°C for 10 min.

Store at 4°C.

Copper (II) Sulfate

Prepare a 20 mM solution.

Sterilize by filtering through a 0.2- μ m pore size filter, in a laminar flow cabinet. Filter into sterile glass bottles in 5 ml aliquots.

Store at room temperature.

Chlorophenol red- β -D-galactopyranoside (CPRG)

Make a 10 mg/ml stock solution. Sterilize by filtering through a 0.2- μ m pore size filter into sterile glass bottles, in a laminar flow cabinet.

Store at 4°C.

Preparation and storage of yeast stocks

Carry out all yeast work in a type II laminar flow cabinet.

Short-term storage (-20°C) - 10X concentrated yeast stock culture

Day 1

Prepare growth medium by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 µl copper (II) sulfate solution to 45 ml minimal medium. Transfer to a sterile conical flask (final volume of approximately 50 ml).

Add 125 µl of 10X concentrated yeast stock from cryogenic vial stored at -20°C. Incubate at 28°C for approximately 24 hour on an orbital shaker, or until turbid. Yeast from a cryovial stored at -80°C can also be used, but may take longer to produce a turbid culture.

Day 2

Add growth medium to two conical flasks (each with a final volume of approximately 50 ml).

Add 1 ml yeast from 24-h culture to each flask.

Incubate at 28°C for approximately 24 hour on an orbital shaker, or until turbid.

Day 3

Transfer each 24-h culture to a sterile 50-ml centrifuge tube.

Centrifuge the cultures at 4°C for 10 min at 2,000 g.

Decant the supernatant, and resuspend each culture in 5 ml of minimal medium with 15% glycerol (add 8 ml sterile glycerol to 45 ml minimal medium).

Transfer 0.5 ml aliquots of the 10X concentrated stock culture to 1.2-ml sterile cryovials.

Store at -20°C for a maximum of 4 months.

Long-term storage (-70°C/-80°C)

Preparation of minimal medium agar slopes (1.5 % agar):

Add 1.5 g bacteriological grade agar to 100 ml minimal medium.

Autoclave, and allow to cool to 50°C before adding the growth medium components:

10 ml glucose,

2.5 ml L-aspartic acid,

1 ml vitamin solution,

0.8 ml L-threonine solution, and

250 µl copper (II) sulfate.

Swirl, and quickly pour into sterile universals (approx. 10 ml per universal). Leave the universals at an angle of approximately 45°, so that the agar sets forming a slope.

Preparation of yeast

Using a sterile loop, take some yeast from a cryovial (stored at either -20°C or -80°C), and spread this over the surface of the slope.

Incubate the slopes at 32°C for 3 days.

Add 1 ml sterile glycerol (100 %) to each universal, and resuspend the cells using a sterile loop.

Aliquot the glycerol yeast suspension to cryovials (0.5 ml per cryovial), and store at -70°C or -80°C.

Store at -70°C or -80°C for a maximum 6 months.

Preparation and storage of chemicals

Glassware must be scrupulously cleaned since contamination may give rise to false positives.

Rinse all glass bottles twice with absolute ethanol (or twice with methanol, and once with

ethanol), and leave to dry.

Weigh chemicals directly into bottles.

Prepare the 17 β -estradiol stock for the standard curve in absolute ethanol, at 2×10^{-7} M (54.48 $\mu\text{g/L}$).

Stock solutions of test chemicals are prepared in absolute ethanol at 2×10^{-2} M or 2 g/L, although this concentration may vary, depending on potency, toxicity etc.

If the chemical is insoluble in ethanol, try another solvent, such as minimal medium. However, certain solvents will melt plastic plates.

Assay procedure

Carry out all yeast work in a type n laminar flow cabinet.

Day 0

Preparation of yeast for assay:

Prepare growth medium by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 μl copper (II) sulfate solution to 45 ml minimal medium. Transfer to a sterile conical flask (final volume of approximately 50 ml).

Add 125 μl 10X concentrated yeast stock from a cryogenic vial.

Incubate at 28°C for approximately 24 hours on an orbital shaker, or until turbid.

Day 1

Serially dilute chemicals in absolute ethanol (100 μl volumes), in a 96-well microtitre plate.

Transfer 10 μl aliquots of each concentration to a 96-well optically flat bottom microtitre plate.

Add 10 μl absolute ethanol (or appropriate solvent) to blank wells.

Leave chemicals in the assay plate to evaporate to dryness.

Prepare assay medium by adding 0.5 ml CPRG to 50 ml fresh growth medium. Seed this medium with 4×10^7 yeast cells (1.8 ml) from the 24-h culture prepared on Day 0. For every 2.5 assay plates, prepare 50 ml assay medium. Add 200 μ l of the seeded assay medium (growth medium containing CPRG and yeast) to wells using a multi-channel pipette.

This 1/20 dilution gives final well concentrations of -

17 β -estradiol - 1×10^{-8} M (2,724 ng/L) to 4.8×10^{-12} M (1.3 ng/L)

Each plate should contain at least one row of blanks (solvent and assay medium only), and each assay should have a 17 β -estradiol standard curve.

Be aware that some chemicals (particularly alkylphenolic chemicals) creep across the assay plate to contaminate adjacent wells, and should therefore be placed on separate plates.

Seal the plates with autoclave tape and shake vigorously for 2 min on a titre plate shaker.

Incubate at 32 °C in a naturally ventilated heating cabinet.

Day 2

Shake the plates vigorously for 2 min, to mix and disperse the growing cells. Return to 32°C incubator.

Day 4

After incubating for 3 days, shake plates (2 min) and leave for approximately 1 hour to allow the yeast to settle. Read the plates at an absorbance of 540 nm (optimum absorbance for CPRG ~575 nm) and 620 nm (for turbidity) using a plate reader.

Leave the plates at room temperature and read later if necessary.

Calculations

To correct for turbidity the following equation needs to be applied to the data:

Corrected value = chem. abs. (575 nm) - [chem. abs. (620 nm) - blank abs. (620 nm)]

Vita

Zunyang Zhao was born in Liuhan Village, Yinan County, Shandong Province, P.R.China. He got his BS in Animal Science in 2000 and MS in Ruminant Nutrition in 2003 from China Agriculture University. He worked one year as an assistant manager in Zhongmu Hualuo Feedmill in Beijing, China.